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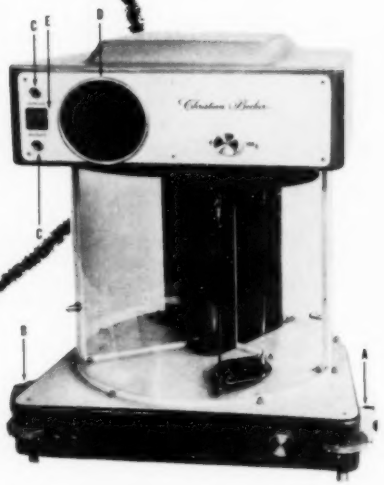

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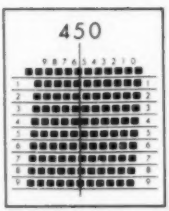

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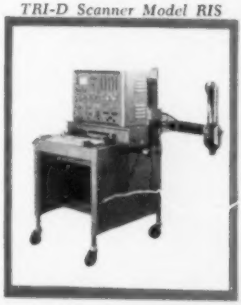

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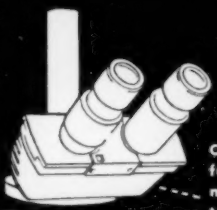

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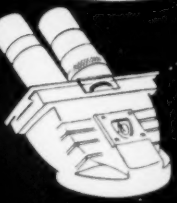

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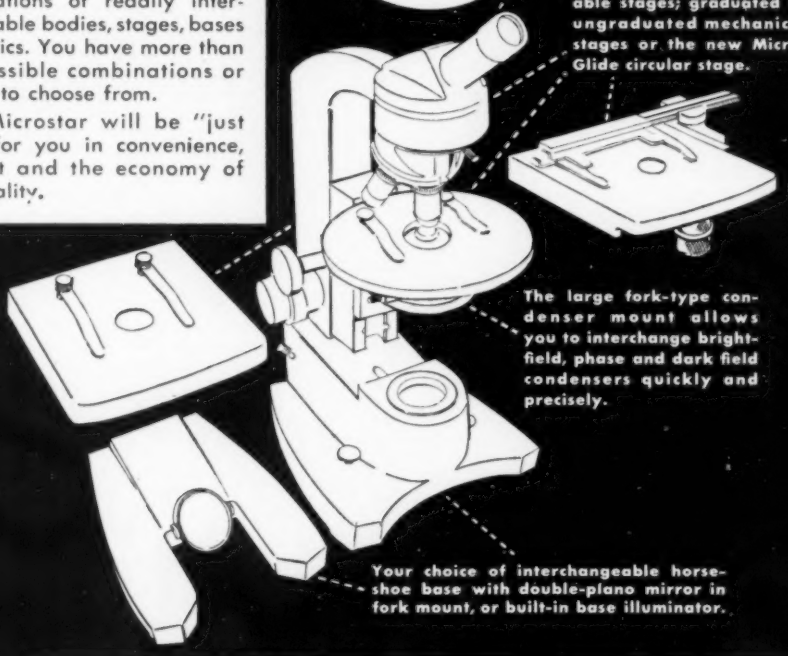

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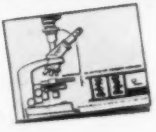

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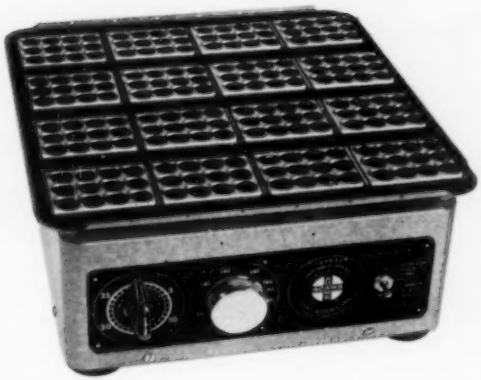

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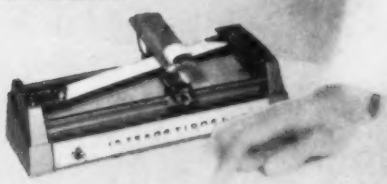

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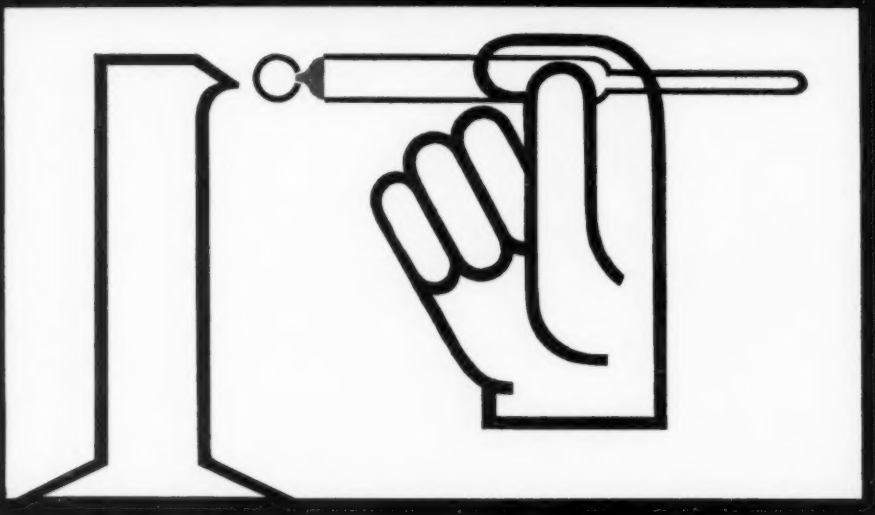

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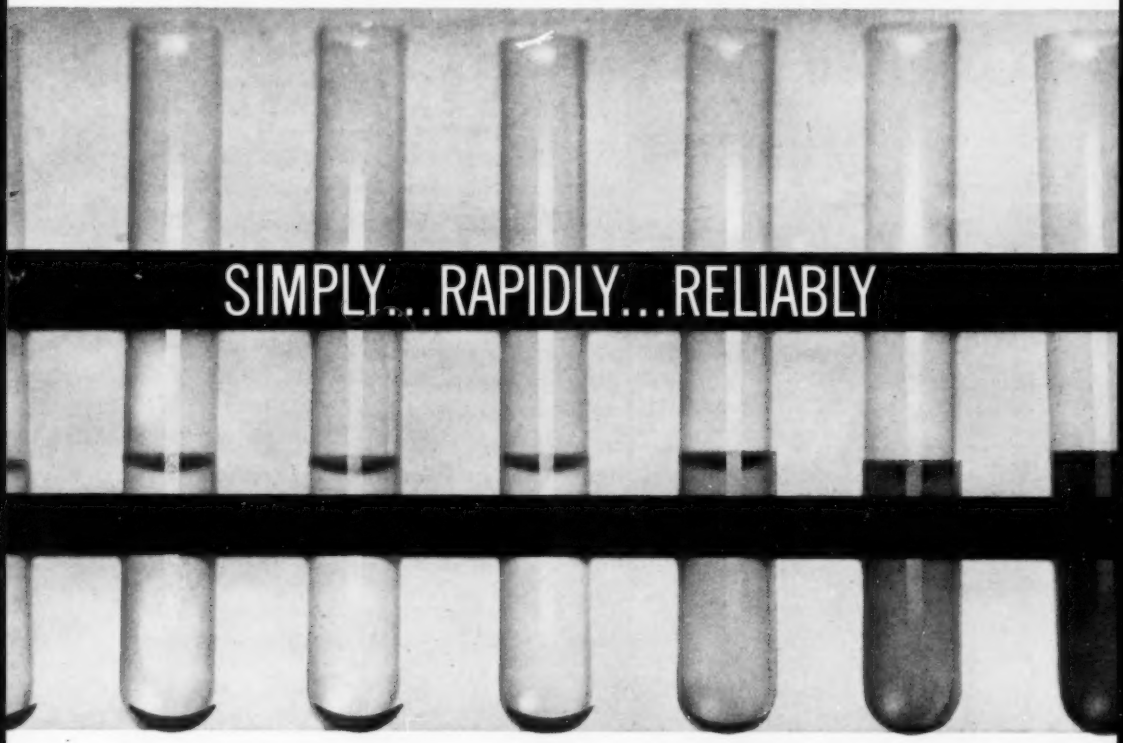

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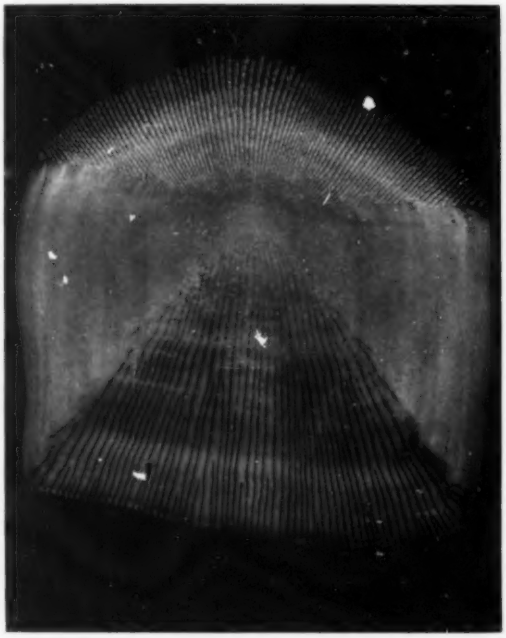

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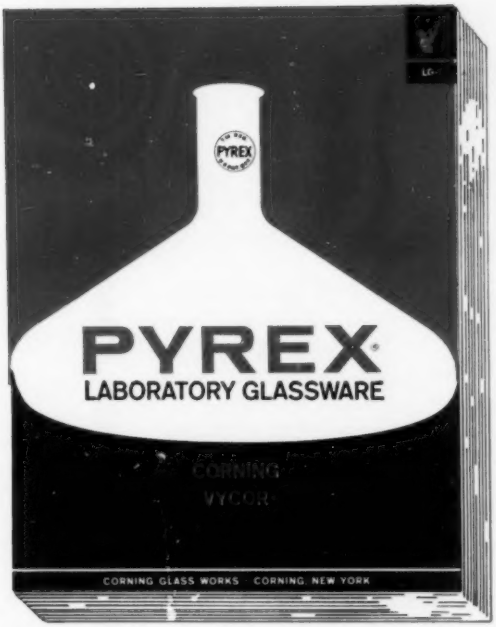

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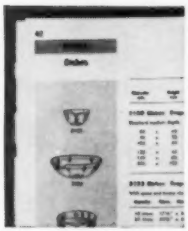

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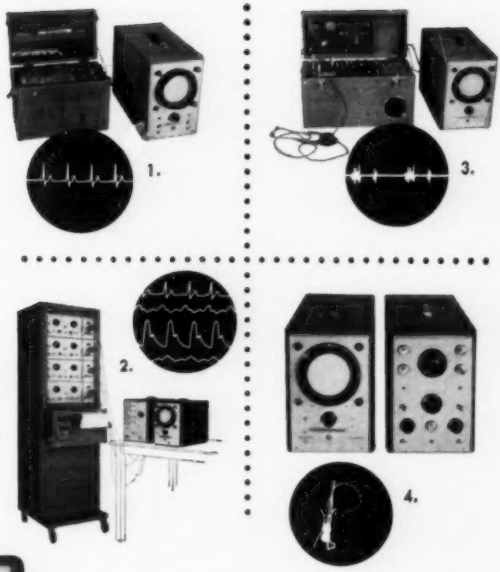

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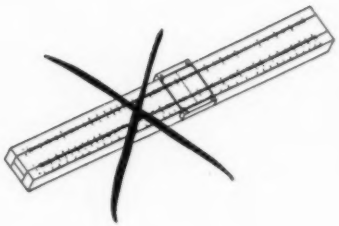

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1. Schiff, F. and Sasaki, H.: Zschr. Immunforsch. 77: 129-139, 1932.
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5. McNeil, C., et al: Am. J. Clin. Path. 28(5): 469-480, (Nov.) 1957.

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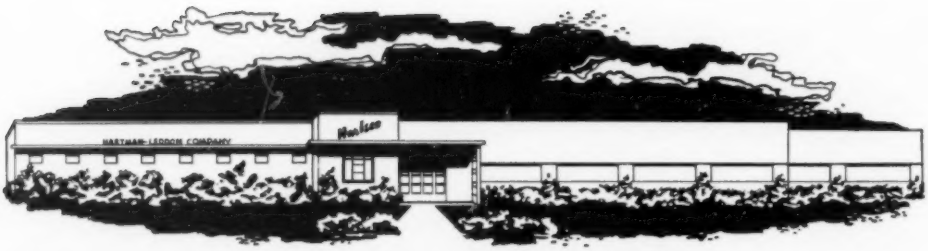

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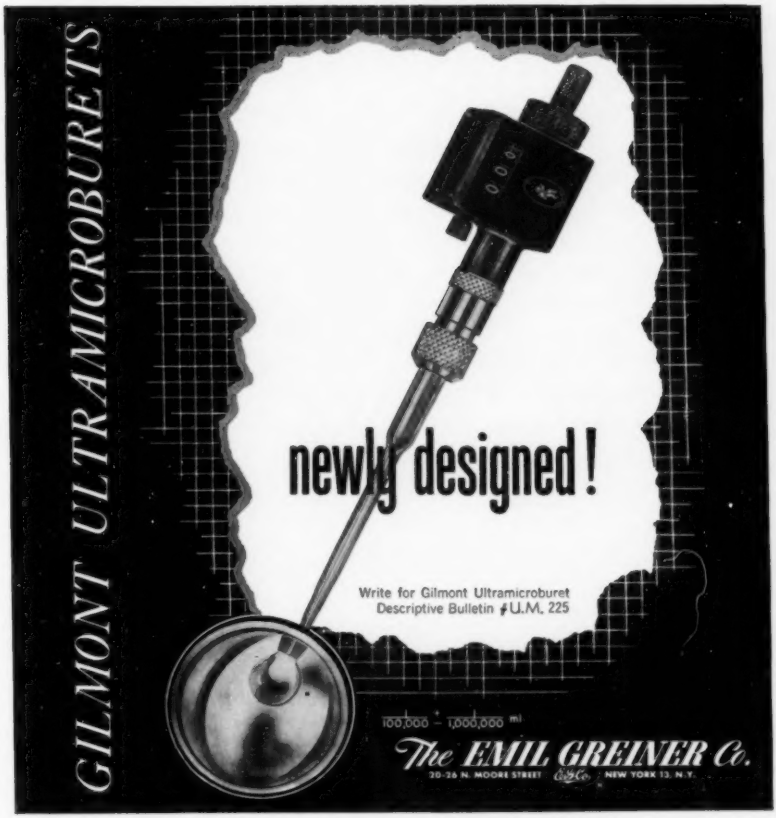
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1. Rein, C. R., Fleischmajer, R., and Rosenthal, A. L.: J. A. M. A. 165:1821, (Dec 7) 1957.
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12. Cooke, R. A.: Personal Communication.
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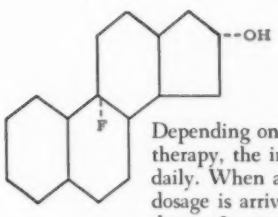

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Goldbloom, A. A., Feinstein, M. A. and Eiber, H. B.: *Am. J. Dig. Dis.*, 22:288, 1955.

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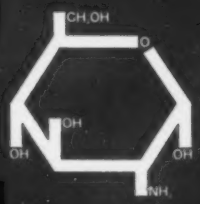

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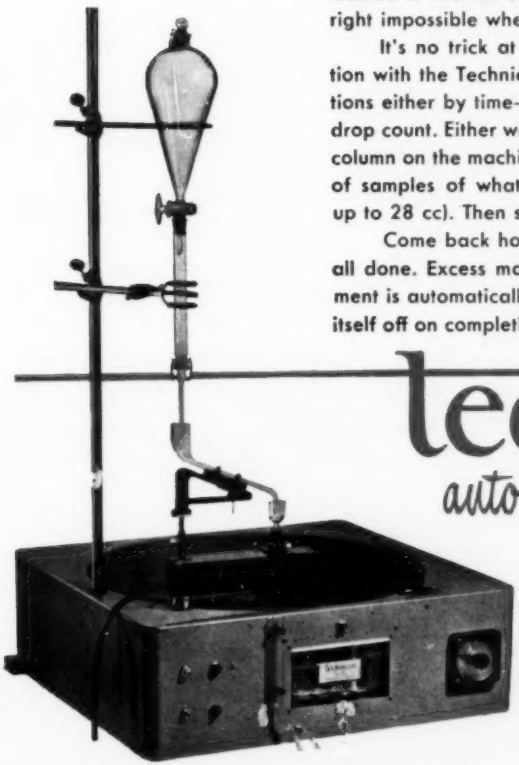

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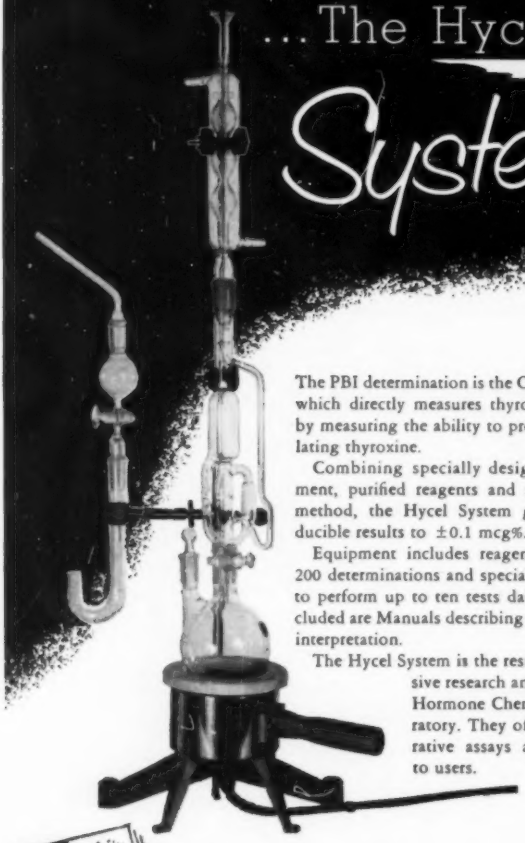

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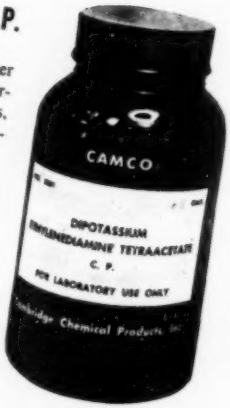

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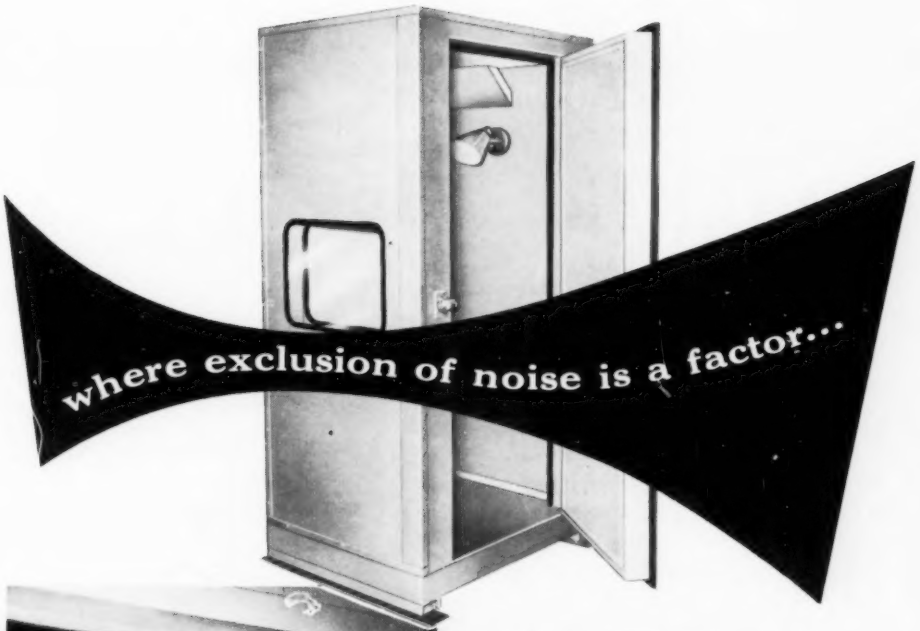

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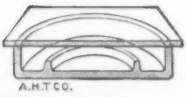

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Edward J. Conway, *Micro-Diffusion Analysis and Volumetric Error, 3rd Ed., 1950* (D. Van Nostrand, New York), includes all literature references up to and including year 1950.

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R. F. Milton and W. A. Waters, *Methods of Quantitative Micro-Analysis,* (Edward Arnold, London) *1955, pp. 214-218.*

Samuel Natelson, *Microtechniques of Clinical Chemistry for the Routine Laboratory,* (Charles C. Thomas, Springfield, Ill.), *pp. 22, 86, 96 and 153.*

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CLINICAL AND EXPERIMENTAL

PLASMA CHOLINESTERASE AND OTHER LIVER FUNCTION TESTS IN HEPATIC AMEBIASIS

GORDON B. MAGILL, M.D.,* AND JOHN H. KILLOUGH, M.D.**
DENVER, COLO.

ALTHOUGH there have been advances in diagnosis and treatment of intestinal amebiasis, recognition of the hepatic form has remained a frequently vexing problem. Klatskin[1] and DeBakey and Oehsner[2] estimated that hepatic amebiasis was found 4 to 7 times more frequently at autopsy than noted clinically. A large amebic abscess of the liver may be quite apparent at times. Fortunately, however, this extreme stage is not common[3] and should be considered an end product of amebic invasion of the liver. As indicated by Sodeman[4, 5] it is the cases with early liver involvement that offer the greatest diagnostic difficulties. Since it is this same group that shows the most promising therapeutic results, early recognition of hepatic amebiasis is of prime importance.

One of the difficulties in the diagnosis of early, or even moderately advanced, hepatic amebiasis is the failure of the usual liver function tests to indicate involvement of that organ.[1, 4-8] With the development of more sensitive liver function tests and more accurate means of determination, it seemed pertinent to study a battery of these tests in patients suspected of having hepatic amebiasis. It is the purpose of this report to present the results of 7 liver function tests performed on a series of 101 patients with amebiasis. Of these, 46 were suspected or proved to have hepatic involvement.

Work performed and report prepared while authors were members of the Department of Clinical Investigations, U. S. Naval Medical Research Unit No. 3, Cairo, Egypt (1950-1953).

These studies were made possible by the cooperation of the Egyptian Ministry of Health and the directors of Abbasieh and Imbaba Fever Hospitals and the Endemic Disease Institute: Dr. A. H. El Ramli, Dr. Abdul Wahid and Dr. A. Halawani, respectively.

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**Present address: University of Missouri, Columbia, Mo.

METHODS AND MATERIALS

Patients.—Cases for study were selected from several Egyptian institutions on the basis of *Endamoeba histolytica* in the stools.* Due to the lack of facilities for the care of women, all patients were men. Since cases were at times selected primarily for the study of dysentery, it should be emphasized that the high percentage of severely ill patients in the present series does not reflect the relative incidence of this type of amebiasis in Egypt. On the contrary, several routine surveys of villages showed a high proportion of asymptomatic, or only slightly symptomatic, amebiasis.[9]

TABLE I. HISTORICAL DATA IN 101 PATIENTS WITH AMEBIASIS

GROUP	NO. OF CASES	AGE MEAN YEARS (RANGE)	DIARRHEA AND CRAMPS			RIGHT UPPER QUADRANT PAIN (PER CENT)
			PAST ONLY (PER CENT)	PRESENT (PER CENT)	TOTAL (PER CENT)	
I. No hepatic involvement	55	27 (16-50)	10 (18)	24 (49)	37 (67)	5 (9)
II. Amebic hepatitis (± ↑ abscess)	41	32 (10-60)	3 (7)	33 (81)	36 (88)	10 (24)
III. Hepatic abscess	5	39 (30-65)	0	5 (100)	5 (100)	4 (80)

TABLE II. PHYSICAL AND LABORATORY FINDINGS

GROUP	LIVER TENDERNESS*				FEVER		W. B. C.		STOOLS		
	0 (%)	1-2+ (%)	3-4+ (%)	TOTAL NO. (%)	NO. WITH FEVER† (%)	MEAN MAXIMAL RECTAL TEMP.	NO. WITH LEUKOCYTOSIS‡ (%)	MEAN	NO. PER DAY (MEAN)	CONSISTENCY (BLOOD, MUCUS, AND DIARRHEA) (%)	NO. WITH TROPHOZOITES WITH INGESTED R.B.C.§ (%)
I	37 (68)	16 (30)	1 (2)	17 (32)	7 (13)	99.8°	11 (20)	7,800	2.6	4 (7)	6 (11)
II	4 10)	22 (54)	15 (36)	37 (90)	28 (68)	100.8°	32 (78)	14,200	7.1	24 (59)	27 (66)
III	0	1 (20)	4 (80)	5 (100)	5 (100)	101.5°	5 (100)	24,200	13.8	4 (80)	4 (80)

*By palpation of liver or percussion or compression of rib cage over liver.
†Greater than 100.0° F. (rectally).
‡W.B.C. above 10,000.
§The balance had cysts only or cysts and trophozoites without ingested R.B.C.

Classification of Cases.—Large surveys of hepatic amebiasis have emphasized the diagnostic importance of liver tenderness.[1, 2, 4, 7] The two other findings most frequently emphasized have been fever and leukocytosis. It was, therefore, decided to classify the patients in the present study on the basis of these 3 criteria. Other causes of liver tenderness, fever, or leukocytosis were excluded, when possible. The distribution of cases was as follows (Tables I and II):

Group I consisted of 55 patients with no definite evidence of liver involvement. Included in this group were 20 patients who demonstrated none of the 3 criteria. The other 35

*Protozoal examinations were performed by D. K. Lawless, H.M.C., and C. P. A. Stome, H.M.C., USN.

subjects showed one of the criteria, usually to a minor degree, but were considered likewise as not having hepatic involvement.

Group II was composed of 41 subjects thought very likely to have hepatic involvement but without demonstrable abscess. All of these patients showed at least 2 of the basic criteria, with 26 (63 per cent) demonstrating that number and the other 15 (37 per cent) evidencing all 3 criteria. Undoubtedly some of the patients in this group had actual liver abscess but could not be classified as such because of recovery without actual demonstration of the lesion. Proctoscopy was performed in 15 patients and revealed mucosal ulcerations.

Group III was limited to 5 patients in whom abscess was unequivocally demonstrated, either by autopsy (3 cases) or by aspiration of exudate (2 cases). Hepatic tenderness, fever, and leukocytosis were present in all cases.

Liver Function Studies.—Plasma cholinesterase determinations were performed by the procedure of Michel[10] with the slight modification of using acetylcholine bromide in place of the chloride salt. Bromsulphalein retention was measured 45 minutes after injection of 5 mg./Kg. of body weight.[11] Serum bilirubin was estimated by the procedure of Malloy and Evelyn.[12] Cephalin flocculation was measured by a turbidimetric modification* of Hanger's procedure.[13] Thymol turbidity was estimated by Shank and Hoagland's modification of Maclagan's technique[14] and zinc and phenol turbidities according to the methods of Kunkel and associates.[15, 16]

Control values for these tests were obtained simultaneously from Egyptians who were apparently in good health. Mean control values and ranges (mean ± 2 s.d.) were as follows: cholinesterase, 0.99 (0.44 to 1.54) pH units (Δ pH/hour); bilirubin, 0.50 (0.00 to 1.00) mg. per cent; cephalin turbidity, 2.8 (0.0 to 5.6) turbidity units; thymol turbidity, 2.6 (0.0 to 5.2) units; zinc turbidity, 13.5 (6.0 to 21.0) units; and phenol turbidity, 20.5 (10.0 to 31.0) units.

RESULTS

Plasma Cholinesterase.—(Table III and Fig. 1). Of the 25 Group I patients tested for this enzyme, 2 (8 per cent) had abnormally low values. The mean value for that group was 0.74 pH units. In contrast, abnormal values were noted in 13 (62 per cent) of 21 patients with clinical evidence of amebic hepatitis (Group II) and in all 3 (100 per cent) of those with proved hepatic abscess (Group III) in whom the test was performed.† The mean values in Group II and Group III were 0.39 and 0.21 pH units, respectively.

Bromsulphalein Retention.—Two (4 per cent) of the 55 patients in Group I had increased B.S.P. retention. The group mean was 1.2 per cent. Increased retention was noted in 7 (17 per cent) of the 41 Group II patients, and in all 5 (100 per cent) of Group III. The means of Groups II and III were 3.3 and 14.8 per cent, respectively.

Serum Bilirubin.—Abnormal serum bilirubin levels were noted in one patient (2 per cent) of Group I; in 2 (5 per cent) of Group II; and in none of Group

*In order to quantitate readings, 48-hour cephalin flocculation tests were read in the spectrophotometer. Only the supernatant portion was read if the samples had flocculated. Utilizing a group of normal controls with negative flocculation tests, a semilogarithmic curve (per cent transmission versus turbidity units) was established in which all negative tests read under 5 units and the highest possible (4+) test would read 25 units (complete flocculation 100 per cent transmission). The equivalent range of flocculation versus turbidity units, then, was: negative flocculation, under 5 units; 1+ flocculation, 5 to 10 units; 2+, 10 to 15 units; 3+, 15 to 20 units; and 4+, 20 to 25 units.

†Subsequent to this study another case of proved amebic liver abscess was investigated. Plasma cholinesterase ante mortem was 0.21 pH/hour. (Personal communication: T. W. Burns, M.D.)

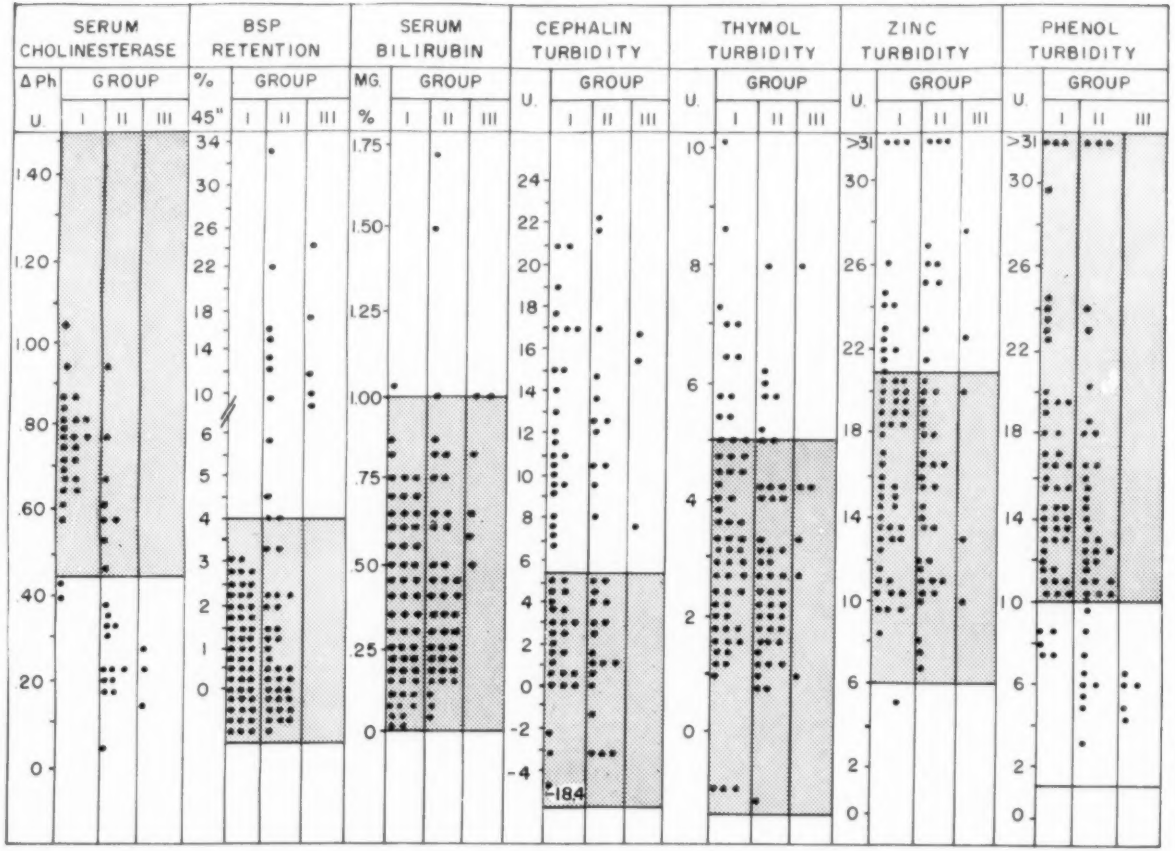

Fig. 1.—Distribution of values of the various liver function tests in patients with no evidence of hepatic amebiasis (Group I) versus those with evidence suggestive of amebic hepatitis (Group II) and those with proved liver abscess (Group III). Shaded areas represent normal ranges of the individual tests.

III. The means of Groups I, II, and II were 0.43, 0.87, and 0.81 mg. per cent, respectively.

Cephalin, Thymol, and Zinc Turbidities.—Cephalin turbidities were abnormal in 48 per cent of Group I, 40 per cent of II, and 100 per cent of III. Abnormal thymol turbidities were noted in 11 per cent of Group I, 10 per cent of II, and 0 per cent of III. As regards zinc turbidity, 22 per cent of Group I were elevated, 24 per cent of II, and 40 per cent of III.

Phenol Turbidity.—Total lipids, as estimated by the phenol turbidity, were depressed (below 10.0 turbidity units) in 5 (9 per cent) of the 55 patients in Group I. Depressed values were noted in 9 (22 per cent) of Group II and in all 5 (100 per cent) of Group III.

Observations During Therapy.—Since the purpose of the present report was to evaluate liver function tests as indicators of hepatic involvement in amebiasis, only observations made before specific therapy have been considered in detail (specific therapy consisted of oxytetracycline, chlorotetracycline, fumagillin, Diodoquin, chloroquine, or emetine, singly or in combination[17-19]). It should be noted, however, that serial determinations during and after therapy yielded additional information regarding the value of the tests. This was particularly true of cholinesterase values in patients who received drugs subsequently shown to be ineffective against hepatic amebiasis. Plasma cholinesterase levels became depressed in an additional 4 patients of Group I during such treatment, making a total of 6, or 24 per cent, abnormal. In Group II, on the other hand, 7 of the 8 who had had normal values before treatment subsequently showed low values during treatment. Twenty (95 per cent) of the 21 patients in Group II, then, demonstrated abnormal cholinesterase levels during the first few weeks of hospitalization. As exemplified by Fig. 2 these abnormal values tended to become normal during or after specific therapy.

CASE REPORTS

In order to illustrate the clinical findings and course in patients considered to have hepatic amebiasis, detailed reports are presented of a patient from each of Groups II and III.

CASE 1.—Amebic hepatitis and probable abscess. A 50-year-old Egyptian man was admitted with a one-month history of bloody diarrhea, anorexia, nausea, vomiting, and abdominal pain (especially right upper quadrant). Apparently the patient had had similar, though apparently less severe, bouts for the previous 10 years. He had been treated for urinary bilharziasis 16 years prior to admission.

Physical examination on admission revealed wasting, dehydration, and a friction rub over the liver in the right eighth and ninth interspaces in the anterior axillary line. Marked liver tenderness with guarding in the right upper quadrant, generalized abdominal tenderness, and hyperperistalsis were also noted. Proctoscopy demonstrated inflammation and ulceration of the rectal mucosa. Proctoscopic and liquid bloody stool specimens revealed numerous trophozoites of *Endamoeba histolytica*, many containing ingested red blood cells. Peripheral white blood cells numbered 37,800 with a shift to the left.

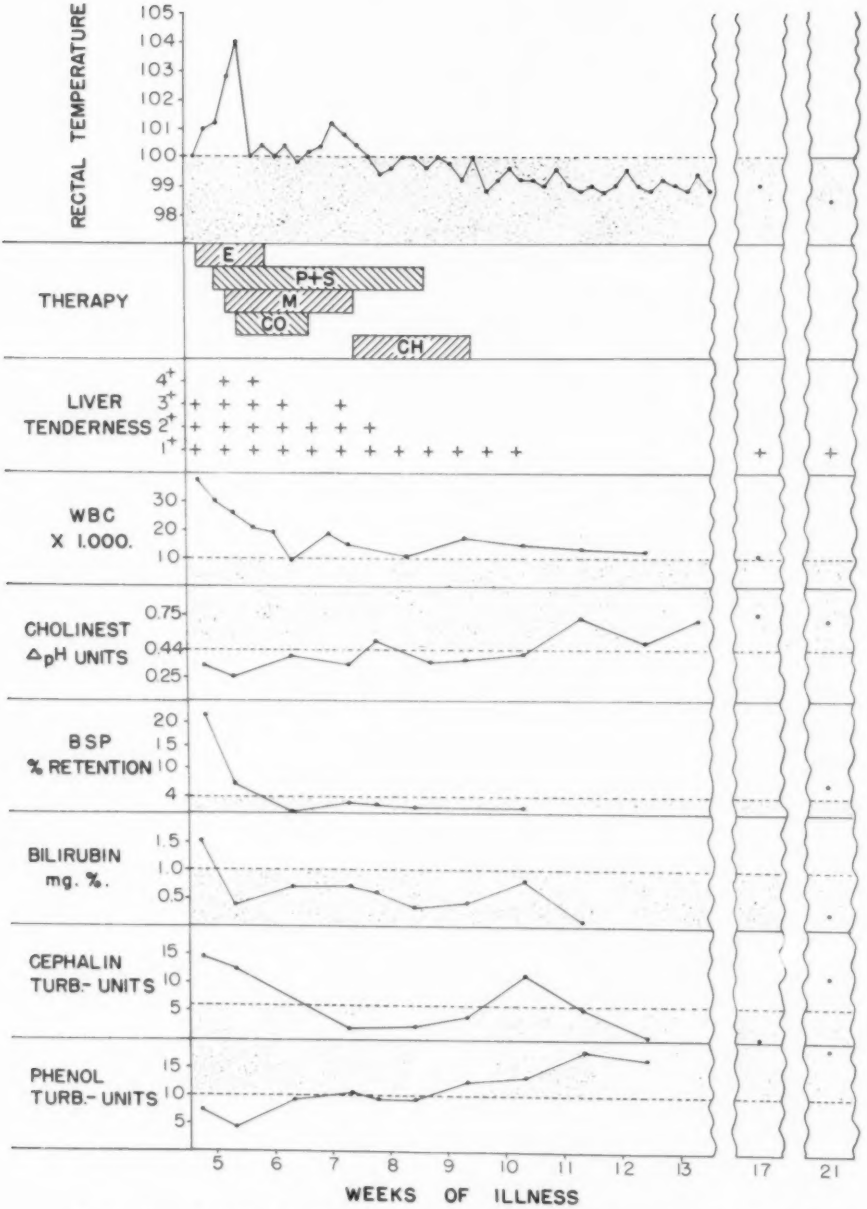

Fig. 2.—Acute amebic hepatitis and probable abscess in a 50-year-old man. E = emetine; P + S = penicillin plus streptomycin; M = milibis; CO = cortisone; CH = chloroquine (Case No. 1).

Liver function tests on admission revealed marked abnormalities (Fig. 2). The patient was given supportive and symptomatic therapy and placed on emetine (0.065 Gm. daily) and erythromycin (3.0 Gm. daily). In spite of this therapy, however, the disease clinically appeared to be progressing. Erythromycin was discontinued; penicillin and dihydrostreptomycin were added to the regimen, followed by Milibis. Nevertheless, the patient continued a downhill course and appeared to be approaching a terminal state. In an attempt to reverse the process, cortisone therapy was instituted (0.1 Gm. daily) and the patient was given several whole blood transfusions. On this combined therapy the patient gradually began to improve. He became more alert and cooperative and fluid balance was restored. During the 10 days of cortisone therapy, liver tenderness and fever diminished; white counts became normal; and liver function tests returned to, or toward, normal limits. Following cessation of hormone therapy there was a slight return of fever, leukocytosis, and liver tenderness, associated with a transient exacerbation of two of the abnormal liver function tests—cholinesterase and phenol turbidity. After the Milibis therapy was terminated, the patient was given a 2-week course of chloroquine (8.0 Gm.). Subsequent recovery was uneventful. Several follow-up examinations during 7 months subsequent to discharge revealed no evidence of recurrence of either colonic or hepatic amebiasis.

Case 2.—Fatal Amebic Abscess. A 65-year-old Egyptian man was admitted with a 3-month history of bloody diarrhea, cramps, anorexia, and weight loss. The patient denied previous episodes of a similar nature, but his history was considered generally unreliable.

Physical examination on admission revealed weight loss, basilar pneumonitis in the right lung, moderate liver tenderness, and generalized abdominal tenderness. Proctoscopy demonstrated ulcerative lesions both of amebic and bilharzial origin. Liver function studies revealed gross abnormalities in the following tests: cholinesterase 0.22 pH units; phenol turbidity 6.2 units; B.S.P. retention 10.2 per cent; and cephalin turbidity 16.7 units. Zinc and thymol turbidities and serum bilirubin were not significantly abnormal. White blood cells numbered 23,000 with a shift to the left.

Initially, the patient made a fairly good response to oxytetracycline therapy with decrease in cramps and diarrhea. Following a 2-week course of this antibiotic he was put on a course of Fuadin therapy for bilharziasis. The patient subsequently suffered a relapse of amebic dysentery, with increased right upper quadrant pain and fever. He followed a progressive downhill course in spite of oxytetracycline and chloroquine therapy and expired on the thirty-second hospital day. Autopsy revealed a large amebic abscess in the diaphragmatic aspect of the right lobe of the liver and moderately advanced bilharzial cirrhosis.

DISCUSSION

In order to compare the findings in the 3 groups of patients and to evaluate the results statistically, it was essential to determine whether factors other than hepatic amebiasis might have caused differences in values among the groups. One possible factor was that many patients had other parasitic infestations. That such co-existent diseases probably did not play a significant role in the differences, however, is indicated by the similar proportions of the groups found to be infested with Bilharzia (53 to 65 per cent) or hookworm (30 to 35 per cent). Another factor investigated was that of age, for a comparison of the mean ages indicated a significant difference between Groups I and II ($P < 0.05$) and between Groups I and III ($P < 0.01$) (Table I). Analysis of the plasma cholinesterase by age showed that the mean value for Group I subjects under 30 years was exactly the same as for those over 30 years (0.74 pH/hour in both cases). It is probable, then, that age was not a factor in the cholinesterase differences among

the groups. Others have noted greater susceptibility to amebic hepatitis and abscess in the higher age groups.[1, 2] The mean age differences of the groups in the present series may represent such an increased susceptibility.

For statistical evaluation of the various liver function tests under study, chi square (χ^2) was determined for the numbers of abnormal versus normal tests in Groups II and III versus Group I.[20] In a comparison of Group I and Group II, plasma cholinesterase was the only liver function test that showed a significant difference (Table III). It is interesting to note that in two other tests, B.S.P. retention and phenol turbidity, chi square approached, but did not reach, significance.

TABLE III. LIVER FUNCTION STUDIES

TEST	GROUP	NO. TESTED	NO. ABNORMAL	(PER CENT) ABNORMAL	χ^2*	P(χ^2)	MEAN	"t"†	P (DIFF. MEAN I)
Cholinesterase	I	25	2	8	–	–	0.74	–	–
	II	21	13	62	12.7	<0.01	0.39	6.26	<0.01
	III	3	3	100	9.80	<0.01	0.21	6.39	<0.01
B.S.P. retention	I	51	2	4	–	–	1.2	–	–
	II	45	7	17	3.57	N. S.	3.3	2.31	<0.05, >0.01
	III	5	5	100	31.8	0.01	14.8	13.0	<0.01
Bilirubin	I	55	1	2	–	–	0.43	–	–
	II	41	2	5	N. S.	N. S.	0.48	0.87	N. S.
	III	5	0	0	N. S.	N. S.	0.81	0.82	<0.01
Cephalin turbidity	I	50	24	48	–	–	6.9	–	–
	II	30	12	40	N. S.	N. S.	6.3	0.36	N. S.
	III	3	3	100	N. S.	N. S.	13.1	1.41	N. S.
Thymol turbidity	I	55	6	11	–	–	3.5	–	–
	II	41	4	10	N. S.	N. S.	3.0	1.31	N. S.
	III	5	0	0	N. S.	N. S.	3.3	0.22	N. S.
Zinc turbidity	I	55	12	22	–	–	17.6	–	–
	II	41	10	24	N. S.	N. S.	17.4	0.15	N. S.
	III	5	2	40	N. S.	N. S.	18.6	0.28	N. S.
Phenol turbidity	I	55	5	9	–	–	16.3	–	–
	II	41	9	22	2.17	N. S.	13.3	2.23	<0.05, >0.01
	III	5	5	100	21.1	<0.01	5.6	2.47	<0.01

N.S. = Not significant.

*χ^2 = Chi Square computed for each of Groups II and III on a fourfold contingency table compared to Group I.

†"t" = Student's "t" test, computed for difference between each of means of Groups II and III compared to Group I.

A comparison of liver abscess Group III to Group I showed significant differences for cholinesterase, B.S.P. retention, and phenol turbidity; all of the abscess patients showed abnormal values for these tests. Cephalin turbidity was also abnormal in these patients, but since it was abnormal in such a high proportion of Group I cases (48 per cent), the difference was not significant.

In order to evaluate group liver function, in contrast to that of individuals, mean values for each of the tests were also analyzed (Table III).* The results further confirmed the disturbance of cholinesterase, total lipids (phenol turbidity), and B.S.P. excretion in the amebic hepatitis patients. It also indicated

*Statistical significance was measured by the "t" test on the basis of the difference between the means.[20]

an abnormal mean bilirubin in the abscess patients, even though none of the patients had significantly abnormal bilirubin levels. Such a seeming discrepancy may indicate some bilirubin retention, but not enough to be useful in the diagnosis of individual cases. The question arises whether the high incidence of abnormal zinc and cephalin turbidities (22 and 48 per cent, respectively) present in Group I may not have been an indication of early hepatic amebiasis even in that group. A similar finding was observed in other patients even months after successful treatment for such diseases as typhoid, typhus, and brucellosis. This fact plus the lack of an increased incidence of these abnormalities in Group II suggest that the frequent abnormal zinc and cephalin turbidities in Group I reflect a basic disease process in our patients, probably involving liver function and gamma globulin production. Among the endemic diseases common in our patients, Bilharzia was the most frequent. That disease is known to cause cirrhosis of the liver and is possibly the main factor in the Group I abnormalities.

It has been demonstrated that malnutrition is often accompanied by depression of serum cholinesterase.[22] Such a state was present in a fair number of the subjects constituting the present series and may have played a greater role in the lower mean cholinesterase of Group I (0.74 Δ pH/hr.) than noted in a series of normal well-nourished Egyptians (0.99 Δ pH/hr.). However, malnutrition did not appear to play a significant role in the cholinesterase differences among the groups under consideration. For example, while some of the normal values observed in Group I were in undernourished patients, one of the lowest values in the series (0.22 pH/hr.) occurred in a well-nourished middle-aged man with liver abscess. Similarly, low phenol turbidity (total lipids) may be associated with malnutrition. As in the case of cholinesterase, however, malnutrition apparently did not play a significant role in the phenol turbidity differences noted among the present groups.

There is general agreement that plasma cholinesterase is produced by the liver and that its measurement is useful as a test of liver function.[21-27] It appears that it is associated with protein synthesis, and changes in cholinesterase levels parallel those of albumin. In regard to its value relative to other liver function tests it appears to be particularly valuable in chronic or severe disease of the liver (e.g., cirrhosis or toxic hepatitis) and useful as a prognostic aid.[21-23, 25] Some investigators state that it is also valuable in acute viral hepatitis, especially when used serially.[21, 24] Although two cases of liver abscess due to actinomycosis were described in the first report of the use of cholinesterase as a test of liver function,[22] we have not found any reports of it in amebic hepatitis or amebic abscess of the liver. The present series indicates a special value of that test in such conditions and suggests that it may be a means to earlier recognition and treatment of significant hepatic amebiasis. It should be emphasized that plasma cholinesterase alone would be of no particular value in differentiating a low-grade amebic process of the liver from other chronic liver disease, since both types would tend to depress the values. Depressed values would be particularly suggestive of liver involvement, however, in cases under treatment for proved

amebiasis. Similarly, it would suggest the possiblity of hepatic amebiasis in the differential diagnosis of right upper quadrant pain, especially when associated with liver tenderness, fever, leukocytosis, and accompanied by other normal liver function tests (bilirubin and thymol turbidity, in particular).

Although there was a general correlation in the present series between the severity of the liver involvement and the degree of cholinesterase depression, one could not use the latter as more than a rough prognostic aid. For example, cholinesterase levels were 0.14 and 0.22 pH/hr. in two patients with fatal liver abscesses, but similar levels were observed in 8 of the 21 patients with amebic hepatitis in whom the enzyme was measured. It was one of the latter who showed the lowest value of the entire series (0.049 pH units).*

Subsequent to the completion of this study, Brem reported on the liver function tests in 10 cases of amebic abscess of the liver. Similar to the present series, cephalin flocculation, thymol turbidity, and bilirubin were unaltered, whereas B.S.P. retention was found in 5 of 8 cases. In addition, that author observed elevated alkaline phosphatase in the same proportion of cases. Unfortunately, alkaline phosphatase levels were not determined in our cases, nor were cholinesterase determinations performed in Brem's series. It would certainly be interesting and worth while to know the levels of both enzymes in cases suspected of having amebic abscess of the liver, particularly in view of the apparently divergent mechanisms by which they become abnormal in liver disease.

SUMMARY AND CONCLUSIONS

1. A series of 101 patients with amebiasis were classified into 3 groups as regards degree of liver involvement. Classification was based primarily on 4 criteria: liver tenderness, fever, leukocytosis, and actual demonstration of abscess. Fifty-five cases were thereby considered to have had no definite evidence of hepatic amebiasis (Group I); 41 cases were classified as having had amebic hepatitis with no demonstrable abscess (Group II); and 5 cases were proved to have had hepatic abscess (Group III).

2. A series of 7 liver function tests was studied in those patients. Plasma cholinesterase was found to be the only test that was significantly abnormal in both Groups II (66 per cent) and III (100 per cent) as compared to Group I (8 per cent).

3. B.S.P. retention and total lipids (phenol turbidity) showed an increased proportion of abnormals in Group II, a trend which became significant in the abscess cases (Group III).

4. Cephalin turbidity showed a high percentage of abnormals in abscess cases (100 per cent) and in the hepatitis group (40 per cent). Because of a large number of abnormals in Group I, however, cephalin turbidity could not be considered of specific value in the present series.

*It is of interest to note that this particular patient probably had a liver abscess and that the only type of amebae found in his stool on repeated examinations was the small race of *E. histolytica*, considered to be nonpathogenic by some authorities.

5. The other 3 tests (bilirubin, thymol, and zinc turbidities) showed no significant trends to indicate hepatic amebiasis.

6. It is concluded that, of the liver function tests studied, plasma cholinesterase was the most useful to indicate involvement of the liver in patients with proved amebiasis. In addition, a depressed value might suggest such a process in the differential diagnosis of right upper quadrant pain, when associated with liver tenderness, fever, and leukocytosis, even in patients without established amebic disease.

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THE BASE BINDING PROPERTY OF THE SERUM PROTEINS WITH RESPECT TO CALCIUM

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RONA and Takahashi[1] first showed that calcium in serum exists in two forms, diffusible and nondiffusible, and suggested that the latter is bound to proteins. McLean and Hastings[2] found that the diffusible form was mostly ionic and the nondiffusible form was mostly bound to the proteins in a reversible combination which obeyed the law of mass action. Some workers[3, 4] believed that almost all of the binding of calcium is with the serum albumin; on the other hand, evidences have been presented to show that the globulins also bind calcium.[5-8] However, the base binding property of the different globulin fractions with respect to calcium in the normal and abnormal sera has not been investigated. An attempt was made to study this aspect.

MATERIALS AND METHODS

Subjects were divided into 4 groups. The groups included normal subjects and cases of multiple myeloma, hyperglobulinemia (exclusive of multiple myeloma), and hypoproteinemia. All these cases had normal serum inorganic phosphate.

The blood was collected under oil. Plasma total and ultrafiltrable calcium were determined in each sample by the Clark-Collip modification of Kramer-Tisdall method.[15] Ultrafiltration was done by the technique described by us previously.[9] Total protein was determined by the biuret method.[10] Paper electrophoresis was used to determine the different fractions of the serum proteins.[11] Nonultrafiltrable calcium was calculated in mM./Kg. H_2O using the McLean and Hastings[2] formula for correction of the volume occupied by the solids and the proteins in the sera. Different protein components were expressed as fractions of the total proteins. The albumin was expressed in terms of mM./Kg. H_2O using the molecular weight of 69,000.[12] The nonultrafiltrable calcium was plotted against the different fractions of the serum proteins on arithmatic graph paper. Curves were determined by the method of least squares. Correlation coefficient and standard error for each group were calculated statistically.[13]

RESULTS

Fig. 1 shows the curve for the albumin and the nonultrafiltrable calcium relationship for all the 4 groups when both were expressed in mM./Kg. H_2O. A definite relationship was noted indicating that albumin binds calcium and the relationship between the two could be expressed as $y = mx+b$, where m is the slope and b the intercept of the curve. In our case, the m was calculated to be 1.23 and b equaled 0.70. It is assumed that the constant b represents the nonultrafiltrable calcium which is independent of albumin in the sera. From the

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above approximately 1.23 moles of nonultrafiltrable calcium is bound to one mole of albumin. A higher intercept b would be obtained if one considered only the hyperglobulinemia group which would indicate that a greater than normal

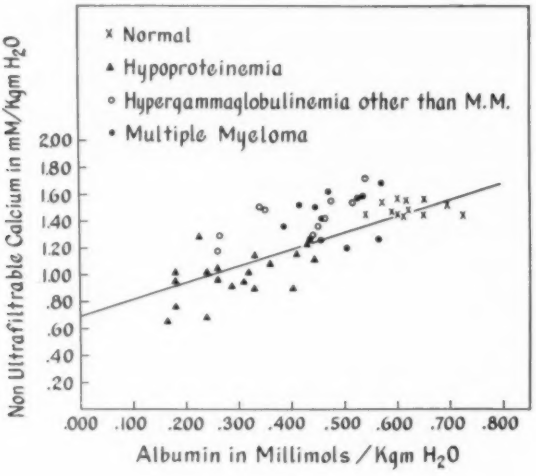

Fig. 1.

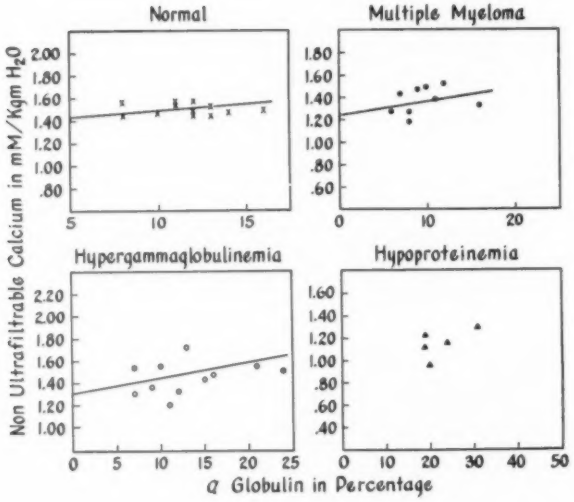

Fig. 2.

amount of nonultrafiltrable calcium is bound to substances in plasma other than the albumin. However, as determined by the slope of the curve, it appeared that the calcium-albumin relationship remained the same in all the groups considered. The correlation coefficient (r) was 0.8 and the standard error (s. e.) was 0.14.

Fig. 2 shows the curve for the alpha globulin and the nonultrafiltrable calcium relationship. Very little or no correlation between the two was observed in all the groups. For Group I, r = 0.3, s. e. = 0.3; Group II, r = 0.5, s. e. = 0.38; Group III, r = 0.43, s. e. = 0.32; and Group IV, r = 0.7, and s. e. = 0.5. This indicates that very little nonultrafiltrable calcium was bound to this fraction or a constant amount was present in this fraction in all the cases.

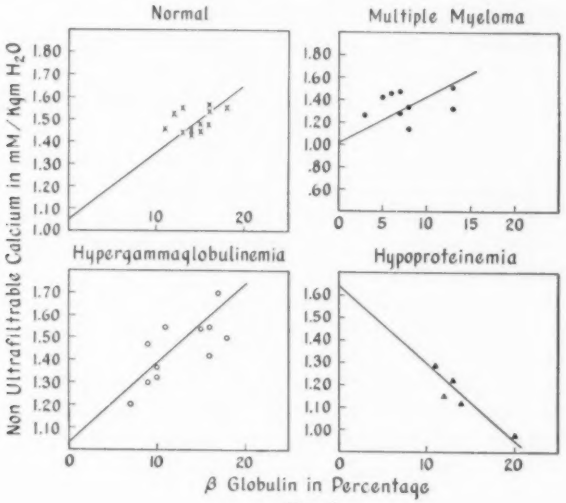

Fig. 3.

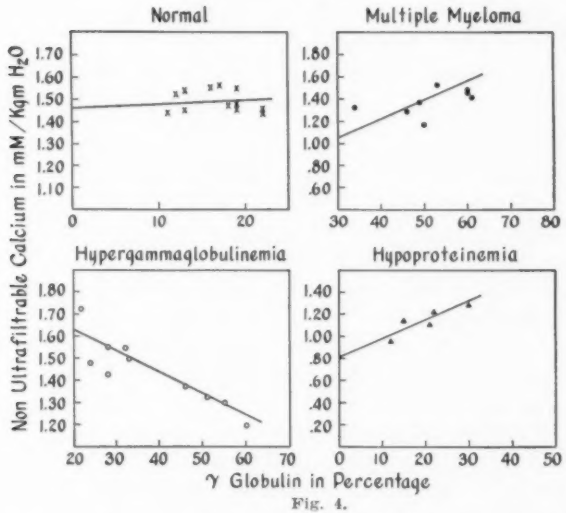

Fig. 4.

Fig. 3 shows the curve for the beta globulin and nonultrafiltrable calcium relationship. Significant positive correlation was observed in Group I (r = 0.7,

s. e. = 0.3) and Group III (r = 0.76, s. e. = 0.32) indicating that this globulin bound calcium. In Group II the correlation was positive but statistically not significant (r = 0.43, s. e. = 0.38). In Group IV, the correlation was negative although statistically not significant (r = –0.75, s. e. = 0.5).

Fig. 4 shows the relationship between the gamma globulin and the nonultrafiltrable calcium. In Group I no correlation was found (r = 0.2 and s. e. = 0.3). In Group II a significant positive correlation was observed (r = 0.8, s. e. = 0.39). In Group III the correlation was negative and statistically significant (r = –0.8 and s. e. = 0.33). This indicated that with increasing concentration of gamma globulin, there was a decrease in binding effect of calcium. For Group IV, r = 0.82 and s. e. = 0.5.

As a gross approximation in the normal, 50 to 55 per cent of the nonultrafiltrable calcium appeared to be bound with the albumin. The rest appeared to be bound with the beta globulin and cephalin as reported by Drinker and Zinsser,[14] whereas the alpha and the gamma globulins accounted for only small amounts of nonultrafiltrable calcium.

DISCUSSION

Although the statistical analysis used here can give only a gross approximation, a few qualitative and quantitative results are worth mentioning. The results indicate that probably each serum protein fraction binds calcium. A good correlation between the alpha and the gamma globulins with the nonultrafiltrable calcium in the normal, was not apparent in our analysis. The reason for this is that normally these fractions comprise only a small part of the total protein pool. Furthermore, the alpha globulin is a heterogenous mixture which partly accounts for the lack of a good correlation. Possibly a better correlation might have been observed if alpha-1 and alpha-2 globulins were plotted separately. A definite negative correlation between gamma globulin and nonultrafiltrable calcium in Group III and probable negative correlation between the beta globulin and nonultrafiltrable calcium in Group IV was observed. The exact significance is not quite clear except that a definite qualitative change in the binding property has occurred. This meant that an increasing concentration of these protein fractions inhibited calcium binding. Recently it has been noted that there is polymerization of gamma globulin in instances of macroglobulinemia and the inhibitory calcium binding effect could be explained if polymerization of the globulins occurred under these circumstances,[16] although the globulins under discussion are not macroglobulins.

Gutman and Gutman[6] by a similar statistical analysis presented the following regression equation: Total calcium = m_1 albumin + m_2 globulin fraction II + m_3 globulin fraction I + b, where b = 5.8 ± 0.2, m_1 = 0.7-0.9, m_2 = 1.0 ± 0.5 and m_3 = 0.1-0.2 (all globulin in excess of 3.0 Gm. per cent total globulin), when all the values were expressed in terms of milligrams per cent. It appears that their globulin fraction I would correspond to the gamma globulin of our Group III. They did not include any cases of multiple myeloma in their study. Globulin fraction II probably represented the alpha and the beta globulins.

Rawson and Sunderman[8] reported a sixfold increase in the binding of calcium with the albumin in one case of multiple myeloma. However, in that case a high total calcium and a decreased ratio between the ultrafiltrable and the nonultrafiltrable calcium was found. In our group of multiple myeloma such decreased ratio between the ultrafiltrable and the nonultrafiltrable calcium was not found. Our results indicate that there is no marked change in the calcium binding property of the albumin in all the groups studied. This would mean that the high protein-bound calcium in cases of multiple myeloma associated with hypercalcemia is the result of hypercalcemia rather than the cause of it. This has been expressed by Gutman and Gutman.[6]

The value for calcium bound when converted to milligrams per cent equals 0.713 mg. of calcium bound to 1 Gm. of albumin. This is in agreement with the value of 0.716 mg. of calcium bound to 1 Gm. of albumin as reported by McLean and Hastings.[2]

Difficulties due to the rapid decrease in the calcium binding properties of the serum proteins as a result of denaturation and many other technical problems still make it difficult to approach the problem more directly.

Calcium is reversibly bound to the proteins according to the law of mass action and not combined irreversibly as is usually implied by the use of the word "bound." In all likelihood, there are quite as many undissociated calcium proteinates as there are discrete proteins in plasma and therefore total plasma nonultrafiltrable calcium moiety should be regarded as the summation of a large number of different protein-calcium complexes.[17, 18]

SUMMARY

1. One mole of albumin appears to bind roughly 1.23 moles of calcium in the normal and hypoproteinemia groups. This relationship appears to remain unchanged in the other two groups associated with hyperglobulinemia.

2. Alpha globulin appears to bind little calcium, if any at all, in all the groups studied.

3. Beta globulin binds some calcium in the normal and hyperglobulinemia due to causes other than multiple myeloma.

4. The gamma globulin in the normal group binds very little calcium. However, in multiple myeloma with increased percentage of myeloma proteins, appreciable amount of calcium is bound to this fraction. In the case of hyperglobulinemia due to causes other than multiple myeloma a marked change in the binding property is obvious. With increasing amounts of this globulin a decreasing binding effect of calcium was observed. Polymerization as a possible explanation has been discussed to account for this behavior.

5. Approximately 50 to 55 per cent of the nonultrafiltrable calcium is bound with the albumin in the normal group. The remaining nonultrafiltrable calcium is in combination with beta globulin and cephalin.

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THE CATHEPTIC CONTRIBUTION TO BLOOD ACID PROTEASE

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With the Technical Assistance of Beatrice Pask and Nancy Trocchio

INDIRECT measures of gastric peptic secretion (blood pepsin and uropepsin) are now widely used to provide an index of gastric secretory activity.[1, 2] Certain discrepancies have prevented a categorical interpretation of the results, however. One of these is the observation that measurable protease activity still is found in the blood following total gastrectomy; such an operation might have been expected to remove all peptic activity.[1, 3] This residual acid protease activity may originate from peptic glands in the esophagus or in the small intestine, or may represent a "cathepsin." The suggestion has even been made that the residual activity is an artefact and related to acid hydrolysis of the substrate.[2] The work to be reported here is concerned with the possible "catheptic" origin of part of acid protease activity.

METHOD

Blood proteolytic activity was measured at a final pH of 2.3 and at 3.7. Blood pepsin or "plasma acid protease" is measured at pH 2.3 by a modification of Mirsky's method[3] and has been shown to reflect the potential peptic secretory ability of the stomach.

Catheptic activity is defined for present purposes as proteolytic digestion of standardized hemoglobin substrate at pH 3.5 to 4.0. Measurement of proteolytic activity at pH 3.5 was first described by Northrop[4] and re-emphasized by Merten.[5] It has the advantage of measuring protease activity at the range in which digestion probably takes place in the stomach. Activity of enzymes in vitro varies with metallic or organic activators, but also (and as importantly) with the substrate and the pH. Discussion of "catheptic" activity in this paper, therefore, must be understood to be applicable only within the specific experimental limits. Even with this narrowing of the meaning of catheptic activity, proteolysis at pH 3.7 may represent the declining activity of pepsin at an increasingly alkaline pH as well as the optimum activity of cathepsin.

Hemoglobin substrate was prepared in the usual way. To analyze for pepsin the plasma was activated at pH 2.3 as previously described. The substrate was prepared for estimating cathepsin by adding to hemoglobin solution, in a 4:1 ratio, a buffer solution prepared by adding 2.64 Gm. of $(NH_4)_2SO_4$ to 77.1 c.c. of acetic acid and making up to 1,000 c.c. with distilled water. This gave a resulting pH of 3.55; the addition of 2 c.c. of plasma makes the final pH 3.7. Analysis was then carried out in duplicate as for pepsin. Acid hydrolysis of hemoglobin substrate at pH 3.7 produces considerable tyrosine in 24 hours (70 units), the amount of which must be accurately taken into account when enzyme activity is evaluated.

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Levels of "pepsin" and "cathepsin" in the same blood sample were studied simultaneously and in duplicate in normal subjects and in patients with gastric hypersecretion or with varying degrees of gastric secretory failure.

TABLE I. BLOOD ACID PROTEASE ACTIVITY IN 71 SUBJECTS WITH VARYING DEGREES OF GASTRIC SECRETORY FUNCTION. EACH PAIR OF VALUES REPRESENTS A SEPARATE INDIVIDUAL

NORMAL SECRETION		HYPERSECRETION		HYPOSECRETION	
pH 2.3	pH 3.7	pH 2.3	pH 3.7	pH 2.3	pH 3.7
580	520	660	570	165	450
530	520	615	495	140	570
510	450	640	545	260	700
565	425	800	530	275	530
400	535	720	530	265	265
320	570	800	475	280	470
350	400	820	625	180	250
360	350	660	450	200	370
410	530	750	600	125	450
365	430	800	500	290	680
350	515	800	580	195	385
400	330	660	520	260	400
315	525	650	460	240	300
415	360	800	570	195	225
470	520	800	640	65	280
360	570	800	470	260	300
300	350	725	430	290	400
415	375	740	550	230	300
390	425	800	530	230	610
400	400	750	445	200	300
440	375	800	515	260	360
300	315	755	600		
440	350	660	450		
450	315				
310	325				
420	400				
340	590				
Average 404	437	673	525	214	458

RESULTS

Table I compares the average cathepsin and pepsin levels in a heterogeneous group of 71 subjects. In subjects with normal pepsin levels there is a slight increase in the activity of the blood measured at pH 3.7; the average pepsin level is *404* units, while the average cathepsin level is *437* units, not a significant change. Blood pepsin levels in a group of patients with duodenal ulcer (or hypersecretion) are considerably higher than the cathepsin levels. There is a drop from the average pepsin value of *673* units to a cathepsin level of *525* units. In patients with gastric secretory failure, the low-pepsin levels are associated with normal cathepsin activity. The average pepsin value is *214* units, while the average cathepsin value is *458* units. The average cathepsin value of the normal subjects and of the group with anacidity is somewhat lower than that in the group with the peptic ulcer or hypersecretion. Table II demonstrates that following total gastrectomy the residual pepsin level increases from *65* units at a pH of 2.3 to *365* units at pH 3.5. The pepsin value drops following subtotal gastrectomy but the cathepsin level is maintained at an almost preoperative level.

TABLE II. CHANGE IN BLOOD ACID PROTEASE ACTIVITY FOLLOWING GASTRIC SURGERY. NOTE THE MAGNITUDE OF CHANGE IN BLOOD "PEPSIN" AND THE SMALLER DEGREE OF CHANGE IN BLOOD "CATHEPSIN"

	DUODENAL ULCER	HEMIGASTRECTOMY	TOTAL GASTRECTOMY
Blood "pepsin" (tyrosine units)	630	385	65
Blood "cathepsin" (tyrosine units)	460	400	365

COMMENT

These results suggest that the major contribution to acid proteolytic activity in the blood above 500 units in duodenal ulcer and in gastric hypersecretion arises primarily from the activity of the peptic glands. The residual acid proteolytic activity in total gastrectomy may be considered a contribution of "cathepsin." Its origin from a source outside the stomach that secretes pepsin seems unlikely. When known amounts of pepsin are added to the blood, measurable peptic acivity diminishes as the pH is made increasingly alkaline; after total gastrectomy residual "pepsin" would have given maximal activity at pH 2.3, with a decrease at pH 3.5, rather than the reverse as found here.

The cathepsin level seems to be completely independent of stomach activity since it is not affected by removal of part of the stomach. The values found after total gastrectomy, which are lower than those found in achylia, suggest that a small part of the activity measured at pH 3.7 may indeed be peptic in origin and that residual pepsin cells remaining after gastric atrophy have been removed by total gastrectomy. Such residual cells can be recognized at gastric biopsy. In any case, pepsin from such residual cells apparently represents only a small portion of the total acid protease activity.

It appears that cathepsin may be affected by factors other than gastric secretory activity. For example, duodenal ulcer patients usually demonstrate a decrease in enzyme activity at a pH of 3.7, but sometimes the decrease is small, and the reason for this is uncertain. In jaundice the cathepsin level is markedly increased out of all proportion to the pepsin levels; the significance of this is under study.

The present results are in general agreement with those of European investigators using different methods. Baur[6] has found the lowest catheptic values in the urine to occur in patients with achylia and chronic gastritis. Following subtotal gastrectomy he did not find urocathepsin changed from normal and interpreted this to mean that the fundus of the stomach secretes urocathepsin. Buchs,[7] however, unable to separate pepsin and cathepsin from each other, has suggested that they are part of the same substance. Pfisterer[8] demonstrated gastric cathepsin and pepsin activity to be elevated in duodenal ulcer preoperatively, but found that following operation, peptic activity decreases while catheptic activity remains unchanged.

West[9] and others have measured "uropepsin" activity at a pH above 4.9 and find no change in this enzyme following subtotal gastrectomy. Studies here related have demonstrated that the pepsin levels drop after subtotal

gastrectomy, but that "cathepsin" is relatively unchanged. We have not measured the so-called "peptic" activity in the urine by West's method but the persistence of unchanged catheptic activity in the blood does suggest that, if comparisons may be validly drawn, the West method of "uropepsin" activity measures a catheptic enzyme which may parallel peptic activity, but which does not necessarily do so.

Certain conclusions appear justified.

1. The proteolytic activity of the blood at pH 3.7, which may be called "cathepsin," is not entirely related to the amount of functioning gastric mucosa, since it is approximately the same in duodenal ulcer subjects, subjects with subtotal gastrectomy, and in patients with gastric secretory failure.

2. The site of catheptic production must, therefore, be outside the stomach.

3. If the results were simply a matter of inhibition of cathepsin by pepsin, patients with subtotal gastrectomy ought to have higher than normal levels at pH 3.5, since there is less pepsin at this pH. This is not the case, however.

These results suggest that measurement of acid protease activity as a rough index of gastric secretory potential is most validly carried out at a moderately acid pH. Discrepancies in the methods used by different investigators may account for varying recorded results.

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ALIMENTARY LIPEMIA AND CORONARY ARTERY DISEASE IN TWO RACIAL GROUPS

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SINCE the suggestion of Moreton[1] that atheromatous lesions may represent lipid deposition secondary to recurrent postprandial elevations of chylomicrons in serum, there has been considerable interest in the significance of alimentary lipemia in the etiology of atherosclerosis. Studies attempting to demonstrate differences in lipemia curves between normal and atherosclerotic individuals have not yielded uniform results. Abnormal elevations in fasting and postprandial plasma turbidity and chylomicron counts have been reported in patients with coronary artery disease by some investigators,[2-6] while others have been unable to demonstrate such differences.[7, 8]

Alimentary lipemia is demonstrable to some degree in all normal individuals after a fatty meal. If the intensity of this phenomenon is related to the development of coronary artery disease, then it might be expected that populations manifesting a higher incidence of this disease would demonstrate a greater degree of postprandial lipemia prior to the development of overt disease.

In recent years, attention has been focused upon the significant differences in the incidence of coronary atherosclerosis among various population groups throughout the world.[9] There is evidence indicating that the susceptibility to this disease among the Negro population in this area is less than in the white.[10, 11] The purpose of the present study was to ascertain whether such a racial difference in the incidence of myocardial infarction was present in this institution and to compare tests of alimentary lipemia in groups of young Negro and white patients.

MATERIALS AND METHODS

Twenty-six white and 26 Negro male hospital patients between 20 and 47 years of age and without evidence of gastrointestinal or cardiovascular disease were studied. These individuals had been following their usual dietary habits prior to hospitalization and were studied during the first week of their admission, while they were subsisting on 3,000 calorie house diets (150 Gm. fat). The studies were begun in the morning following a 10-hour fast. Two control samples of venous blood were drawn into heparinized syringes through indwelling needles. Following this, each subject was given 100 ml. of 10 per cent cream per square meter of body surface area, and hourly plasma specimens were taken. No other food was allowed during the 6-hour observation period. Smoking was interdicted and activity was restricted to bed or chair rest. Optical densities were read against a water blank in a Beckman DU spectrophotometer at a wave length of 650 mμ after the plasma was separated by centrifugation at 2,500 r.p.m. for 15 minutes.

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The control and hourly values were plotted linearly and the areas under the curves derived by planimetric integration. The group means for control and hourly values, as well as areas under the curves were compared statistically according to methods outlined by Snedecor.[12]

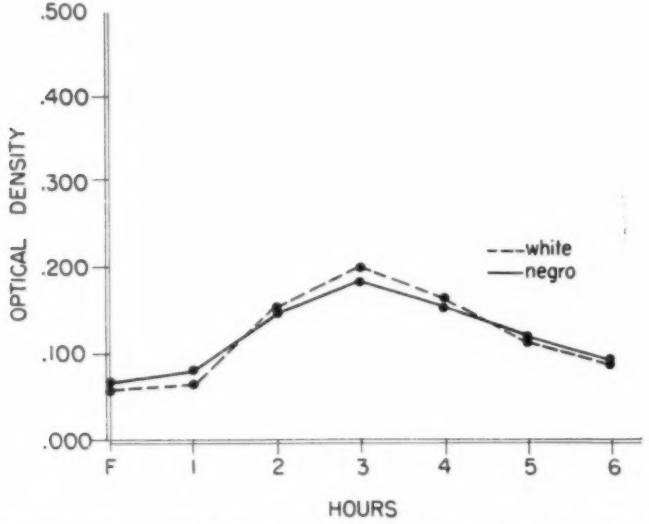

Fig. 1.—Mean plasma optical density curves in white and Negro subjects. Optical density is expressed as Beckman reading. F represents fasting level.

RESULTS

An estimate of the relative incidence of acute myocardial infarction among white and Negro male patients in this area was made from an analysis of all admissions to the Durham V. A. Hospital from the time of its opening in April, 1953, to December, 1956. Two hundred and eighty cases of documented (electrocardiogram and/or necropsy) myocardial infarction were admitted during this period. The incidence of this disease, corrected for the relative admission rate, was found to be 2.2 white to 1 Negro.

The results of the tests of alimentary lipemia are summarized in Table I and Fig. 1. When the hourly mean optical densities are compared, no significant differences between the white and Negro groups were noted. Similarly the mean values for the areas under the lipemia curves were not significantly different. In an attempt to distinguish a possible difference in the occurrence of individual abnormal responses between the two groups, those subjects having 2 or more hourly levels greater than the mean plus 2 standard deviations were separated. Three white and 4 Negro patients fell into this group. Under the conditions of the present report and with the criteria employed, the 2 groups of patients behaved as if they were culled from the same population.

A considerable variability in the degree of serum density following the standard meal was observed between individuals in each of the groups. This was manifest in the large standard deviations of the hourly mean values.

Despite these large differences among individuals, a fair degree of reproducibility of serial lipemia curves (4 to 5 days apart) in the same subject was found (Fig. 2).

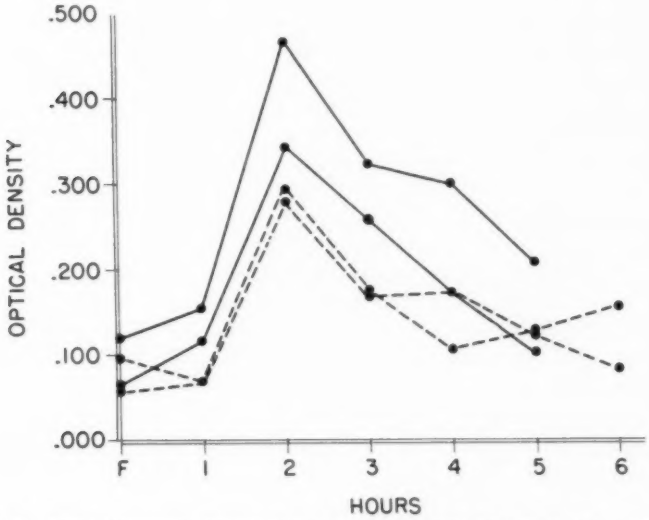

Fig. 2.—Serial alimentary lipemia curves in two subjects performed 4 (- - - -) and 5 (———) days apart.

TABLE I. FASTING AND POSTPRANDIAL PLASMA OPTICAL DENSITY* IN WHITE AND NEGRO SUBJECTS

	NO.	AGE	F	HOURS 1	2	3	4	5	6	AREA UNDER CURVE (CM.²)
White	26	33.2 (± 7.2)	0.060 (± 0.021)	0.065 (± 0.029)	0.153 (± 0.074)	0.202 (± 0.089)	0.166 (± 0.087)	0.117 (± 0.074)	0.091 (± 0.033)	27.2 (± 15.1)
Negro	26	34.0 (± 5.8)	0.066 (± 0.015)	0.080 (± 0.048)	0.150 (± 0.068)	0.187 (± 0.144)	0.156 (± 0.061)	0.120 (± 0.056)	0.094 (± 0.012)	24.7 (± 16.7)

*Units represent Beckman readings. Standard deviations are included in parentheses. F represents fasting level.

DISCUSSION

Although overlap with the responses of a normal population has been found, unusual prolongation or elevation of postalimentary lipemia as judged by serum density and chylomicron counts have been reported in patients with established coronary artery disease. If these differences are a reflection of a basic alteration in lipid metabolism which when operating over many years results in the atherosclerotic lesion, this phenomenon might be expected to be demonstrable in younger individuals predisposed to the development of this lesion.

The groups presented here are representatives of a population in which the incidence of proved myocardial infarction in Negroes is 45 per cent of that in the Caucasians. Although this difference in incidence is not as great as that

observed in other parts of the world, it was nevertheless thought sufficiently large to serve as a basis for experimental studies. No significant differences between these representative groups were found in their responses to the test of alimentary lipemia described above.

Moreton suggested that chylomicrons per se might be an important factor in determining the widespread prevalence of the atherosclerotic lesion, operating much in the same manner as do colloidal particles in animal studies.[1, 13] Subsequent investigators have implicated an intensified degree of alimentary lipemia in the pathogenesis of this process. The present data, while not illuminating further whether lipid particles are injurious to the arterial wall, are not consistent with the view that intensification of postalimentary lipemia plays a significant role in this process.

The prolonged or elevated lipemia curves found by others in cases of myocardial infarction remain an interesting and unexplained phenomenon. The relationship of this finding to the pathogenesis of atherosclerosis is still uncertain. Factors such as stress, diminished physical activity, and altered dietary habits are still to be investigated as possible alternate explanations for these findings.

SUMMARY

Six-hour postalimentary plasma turbidity curves were studied in 26 white and 26 Negro patients without evidence of coronary artery disease. The relative incidence of documented myocardial infarction in the population from which this group was selected is 2.2 white to 1 Negro. No differences in the degree of alimentary lipemia or in the number of individual abnormal responses were found. These data are not consistent with the view that intensified alimentary lipemia is a significant predisposing etiologic factor in coronary artery disease.

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FURTHER STUDIES ON THE ERYTHROCYTE CONCENTRATION OF POTASSIUM IN CONDITIONS OF HYPONATREMIA AND HYPERNATREMIA

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INTRODUCTION

IN A PREVIOUS report, evidence of a relation between the concentrations of erythrocyte K and plasma Na in a variety of clinical states of hyponatremia and hypernatremia was presented.[1] However, determinations were made only of K per volume of cells, and it could not be stated whether the changes found were due to actual gain or loss of cellular K or were secondary to altered cellular hydration. Accordingly, additional studies were made to elucidate this point. The findings indicate that in the presence of hyponatremia, the red blood cell may gain water and lose K, whereas in hypernatremia there may be a gain of cellular K. Furthermore, the changes found appear to be related to the maintenance of isotonicity between cell and plasma.

MATERIALS AND METHODS

Observations were made on 57 adult subjects placed in 4 groups according to the plasma Na concentration. All subjects were selected at random, and grouping was made solely according to the plasma Na concentration. Group I included 21 normal persons with a mean plasma Na concentration of 142 mM./L. (range 137 to 145 mM./L.). In Group II there were 9 patients with normonatremia but with abnormal conditions, including one each with chronic pyelonephritis and uremia, uterine fibroids, pregnancy, hyperparathyroidism, osteomalacia, and 2 each with skull fracture and induced hyperadrenocorticism. The mean plasma Na concentration was 142 mM./L. (range 138 to 145 mM./L.). In Group III there were 19 patients with hyponatremia with a mean plasma Na concentration of 116 mM./L. (range 86 to 131 mM./L.). There were 7 instances of hepatic coma, 5 of malignant hypertension with cardiac failure, and one each of hepatic cirrhosis without coma, chronic pyelonephritis with uremia, "salt-losing" nephritis, and essential hypertension without cardiac failure. Group IV consisted of 6 patients with plasma Na concentration averaging 165 mM./L. (range 153 to 171 mM./L.). Primary water deficit was probably the cause in all. In 3 patients marked dehydration developed as a result of simple water deprivation. One patient developed hypernatremia during the diuretic phase of acute renal failure, one following the administration of a tube-feeding mixture high in protein content, and one following therapy of diabetic acidosis.

Significant acidosis or alkalosis was not present in any of the above patients at the time blood was obtained. However, 2 additional patients with respiratory acidosis and normonatremia were studied, and the findings will be commented on. Lipidemia was also not present in any case. Mild anemia was observed due to uremia in 3 instances, and blood loss in 2.

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Venous blood was drawn with stasis of less than 2 minutes duration. Stasis of this duration has been shown to have minimal, if any, effect on the erythrocyte contents of water and K.[2, 3] The samples were placed in heparinized vials, and aliquots were immediately centrifuged for plasma separation. The following determinations were made on plasma and whole blood: water by drying at 105° C. for 24 hours, specific gravity by weighing 5.0 c.c. aliquots, hematocrits by centrifuging Wintrobe tubes at 5,000 r.p.m. for 30 minutes, and Na and K by flame photometry using a Weichselbaum-Varney spectrophotometer. In calculating hematocrits, no correction was made for trapped plasma. The amount of plasma trapped is estimated to be 2 per cent or less providing the cells are of normal shape.[4] As no hematologic disorder other than mild normocytic anemia was present in the current study, it was assumed that the amount of plasma trapped was small, and that errors resulting in calculating erythrocyte data would be nullified between groups. Calculations of the plasma Na concentration per plasma water were made using the plasma specific gravity to convert plasma water by weight to volume. The erythrocyte concentrations of water and K were calculated using the plasma and whole blood concentrations, specific gravities, and hematocrits. Analysis of duplicate samples yielded agreements within the following ranges: Na per plasma water 2.0 per cent, water per cell solids 2.0 per cent, K per cell solids 2.5 per cent, and K per cell water 2.5 per cent.

TABLE I. ERYTHROCYTE COMPOSITION IN NORMALS AND THOSE WITH ABNORMAL PLASMA NA CONCENTRATIONS

(Values are given as mean ± standard deviation)

	PLASMA	ERYTHROCYTES		
	NA	H_2O	K	K
	/L. H_2O	/100 GM. SOLIDS	/100 GM. SOLIDS	/L. H_2O
GROUP	(mM.)	(GM.)	(mM.)	(mM.)
I	153.0	199.0	26.0	131.0
Normals (21)	±2.8	±9.1	±1.5	±5.5
II	152.0	203.0	26.1	129.0
Normonatremia plus disease (9)	±1.66	±7.9	±2.27	±9.02
III	124.0	226.0	24.1	107.0
Hyponatremia (19)	±11.7	±13.2	±2.9	±11.7
P		<0.01	<0.01	<0.01
IV	175.0	190.0	29.1	153.0
Hypernatremia (6)	±6.4	±4.3	±2.3	±10.9
P		<0.05	<0.01	<0.01

RESULTS

In Table I are listed the mean erythrocyte concentrations of water and K found in the 4 groups. The values are given in reference to cellular solids as well as water. The solids are assumed to represent cellular stroma and consequently are utilized as a reference base to determine if the cell has gained or lost water or K. The plasma Na concentration is expressed in terms of plasma water.

The erythrocyte composition found in the normals is comparable to that reported by others.[5, 6] In Group II (patients with normonatremia but with abnormal conditions), the mean cellular concentrations of water and K expressed per solids were not different from those of Group I. In Group III with hyponatremia, the mean plasma Na concentration was decreased to 124 mM./L. of plasma water. The mean cellular water content was significantly increased,

and the concentration of K expressed per solids or water was significantly decreased. In the patients with hypernatremia (Group IV) the mean cellular water content was possibly decreased ($P < 0.05$). Expressed per solids the K concentration was significantly increased.

Not included in Table I are 2 patients with normonatremia and respiratory acidosis. In these the cellular water contents were 220 and 243 Gm. per 100 Gm. of solids, both values greater than the normal range. It is of interest that the K concentrations per solids were 27.7 and 33.4 mEq., respectively, these values also being beyond the normal range. Consequently, the concentrations of K per cellular water were within the normal range. The increase in cellular water may have been related to an elevation of CO_2 tension.[7]

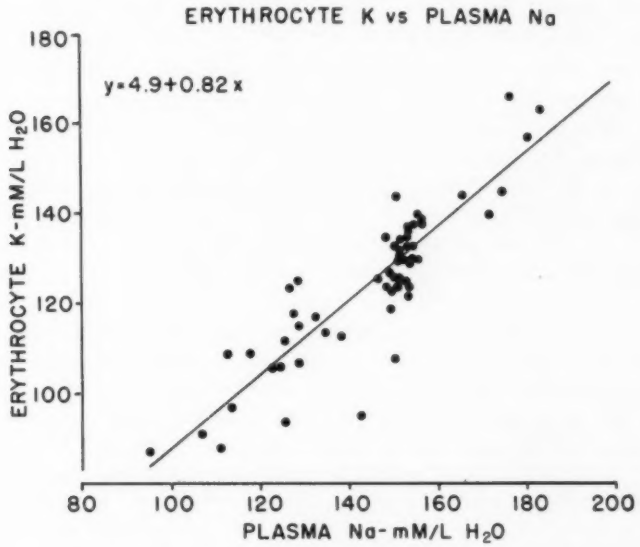

Fig. 1.—The regression of erythrocyte potassium on plasma sodium per liter of water.

In most of the patients in Group III with decreased plasma Na concentration it was felt that the hyponatremia was of the type associated with chronic illness rather than that caused by Na deficit. However, in 4 instances it was believed that Na depletion or water intoxication was partly responsible for the hyponatremia. Evidence for this belief evolved from the immediate beneficial clinical and chemical responses that occurred following Na therapy. It is noteworthy that in these 4 patients the concentrations of K per solids (range 18.8 to 21.8 mM.) determined at the time of Na depletion or water intoxication were the lowest found in the group.

The regression of erythrocyte K concentration per cell water on plasma Na concentration per plasma water is depicted in Fig. 1. The relation is significant ($P < 0.01$) when expressed in linear fashion.

DISCUSSION

The finding that the erythrocyte will gain water in vivo in the presence of hyponatremia confirms for man older observations made in animals with experimentally induced hypotonicity.[8, 9] Other evidence that this phenomenon occurs in man is also apparent from the work of Krainin and associates who utilized this property of the erythrocyte as an indirect method of determining the plasma Na concentration.[10]

Although the cellular water change in the present study was significantly related to the plasma Na concentration, the scatter of data was so marked as to indicate that factors other than the plasma Na concentration per se were concerned with the hydration of the cell. For example, it has been suggested that alterations of plasma Na concentration may not necessarily be accompanied by corresponding changes in plasma osmolality.[11] However, others have demonstrated a dependence of serum osmolality on the plasma Na concentration when appropriate corrections are made for substances such as urea and glucose.[12] Since these substances readily cross the erythrocyte membrane, it is likely that a change in the plasma Na concentration will alter the osmotic forces on the exterior of the erythrocyte. Second, it is possible that in certain conditions metabolic derangements within the cell may lead to changes in the amounts of water and ions available for osmotic activity. Finally, there is also the possibility that the cell may actually lose or gain osmotically active substances.

It is of interest that the 4 patients with hyponatremia who responded favorably to Na therapy were those in whom the greatest losses of cellular K were found. There is evidence from numerous studies that the erythrocyte may lose K in states of K deficiency.[13] Although it cannot be stated with certainty that K deficiency was not present in these 4 subjects, there was no evidence of this condition as gauged by serum K concentration or clinical state. In one patient, serial studies revealed a rise in erythrocyte K concentration following the administration of Na. Consequently, the observations, though limited, suggest that Na depletion or water intoxication might act as a stimulus for the cellular release of K. In support of this concept are the studies of McCance, wherein a loss of erythrocyte K was found in the course of experimental Na deficiency in man.[14]

The decrease in cellular water content encountered in those with hypernatremia was less in relation to elevation of the plasma Na concentration than was the converse found in the hyponatremic group. However, the gain in cellular K was such that the relation of cell K per water and plasma Na was maintained. This finding is not wholly in agreement with that of Eisenman and associates who studied erythrocyte composition before and after the administration of hypertonic Na solution to patients with dehydration.[15] They presented evidence that base may migrate in or out of the cell. However, because the results were not always predictable, they felt that the changes were connected with metabolic rather than osmotic activity.

In studies of K deficiency in man, an increased cellular Na content has been found accompanying a decrease of erythrocyte K.[16, 17] In the present study no such rise in Na was found. Accordingly, the high significance of the regression shown in Fig. 1 is in keeping with evidence from direct cryoscopic measurements that the osmotic activity of the red cell mass is closely related to that of plasma.[18]

SUMMARY

Studies have been made of the erythrocyte contents of water and K in conditions of altered plasma Na concentration. In hyponatremia the cells gained water and lost K. In hypernatremia there was a gain of cell K and a possible loss of water. The erythrocyte K concentration per cell water was related to the plasma Na concentration in a manner to suggest that the changes in cell composition were directed toward the maintenance of isotonicity between cell and plasma.

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CHOLESTEROL ESTER SYNTHESIS AND HYDROLYSIS OF SPLENIC TISSUE IN LYMPHOMA

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EARLIER reports from this laboratory have shown abnormalities of content and partition of cholesterol in serum and tissue of patients with lymphoma.[1, 2] The total cholesterol is low in serum, high in splenic tissue, while cholesterol esters were considerably decreased in the splenic tissue. The accumulation of cholesterol in lymphoma tissue is probably related to the similar behavior of cholesterol-carrying beta globulin; the defect of esterification remains unexplained. This article presents studies on the cholesterol synthesis and hydrolysis of splenic tissue from normal and lymphoma patients and throws some light on our previous findings.

MATERIALS AND METHODS

Spleens from 5 normal individuals and 6 lymphoma patients were studied. Normal spleens represented autopsy material in 3 cases, surgical specimens in 2. Autopsy material was obtained within 2 hours of death. All lymphoma spleens were surgical specimens. Four of the surgical and 3 of the normal specimens were frozen immediately in liquid nitrogen and stored at –20° C. In other instances, the entire spleen was quickly washed free of blood with cold saline solution and frozen at –20° C. until used. All patients were in relapse when studied. Of them, 4 had received no treatment. Results were uniform in both groups, indicating the independence of our findings from therapeutic procedures.

Preparation of Extract from Splenic Tissue.—At the time of study, tissue was thawed in an icebox. After rinsing (3 times) with cold saline solution, tissue was grounded with sand in a mortar after adding an equal amount in weight of 0.9 per cent NaCl solution. The ground material was centrifuged at 1,000 r.p.m. for 20 min. at 4° C. and the supernatant strained through gauze for immediate assay. Each tissue homogenate contained 0.1 Gm. of original tissue per milliliter.

Assay of Esterification and Hydrolysis.—Esterification of cholesterol and hydrolysis of cholesterol esters were studied. Assays were carried out in 25 ml. glass-stoppered test tubes. A mixture of approximately 50 mg. of cholesterol (Merck reagent) and 0.5 ml. of oleic acid at pH 6.1 was used to study esterification[3]; hydrolysis of esters was studied using 50 mg. of cholesterol oleate at pH 6.6. Cholesterol oleate was prepared by heating one mole of cholesterol with two moles of oleic acid for 3 hours under reflux at 200° C.[4]

Substrates were prepared in 25 ml. tubes by adding 50 mg. of cholesterol (or ester) to 0.5 ml. of oleic acid, 10 c.c. of phosphate buffer, and 1 ml. of sodium taurocholate 14 per cent.[5] This solution was completely homogenized by shaking in incubator at 37° C. for 1 hour.

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One milliliter of tissue homogenate was then added and the test tubes again were continuously shaken in an incubator at 37° C. Samples of 0.5 ml. were removed from the test tubes at 0, 6, 12, and 24 hours. Free and total cholesterol were determined in each sample by a modification of the original Schoenheimer and Sperry method, as proposed by Chaney.[8] All calculations were based on the increase or decrease in free cholesterol during the incubation period. Esterified cholesterol was calculated by difference between values for total and for free cholesterol.

Determination of Cholesterol.—Two and one-half milliliters of 3:2 alcohol-ether mixture were blown into glass-stoppered test tubes of 10 ml. capacity, containing 0.5 ml. of each enzyme digest sample. Test tubes were allowed to stand for 10 minutes, centrifuged quickly, and suitable aliquots of the supernatant were then transferred to 15 ml. centrifuge tubes. All aliquots were evaporated to dryness at 60° C. by means of a gentle stream of air and redissolved in 3 ml. of 1:1 acetone alcohol mixture. Samples for determination of total cholesterol were hydrolyzed by adding two drops of 30 per cent aqueous KOH at 60° C. for 30 minutes; then made acid to phenolphthalein with 15 per cent acetic acid. One milliliter of 0.5 per cent digitonin in 50 per cent alcohol was added to all tubes, which were then incubated at 37° C. for 3 hours. The digitonide was well packed by centrifugation, supernatant carefully decanted, and precipitate washed with 3 ml. of anhydrous ether. Mixture was again centrifuged and the ether decanted. Precipitates were dried at 60° C. and dissolved in 0.5 ml. of glacial acetic acid. Three milliliters of chloroform were added and the stoppered tubes warmed to 37° C. in thermostatic incubator. One milliliter of color reagent was then added to each tube. After exactly 10 minutes at 37° C., tubes were transferred to an ice bath for another 10 minutes and then read in Coleman colorimeter Model 63 against a reagent blank at 660 millimicrons.

TABLE I. CHOLESTEROL ESTER SYNTHESIS AND HYDROLYSIS IN EXTRACTS FROM NORMAL SPLEENS: FRESH AND FROZEN TISSUE.*

CASE	TISSUE	TOTAL CHOLESTEROL (MG. %)	ESTERIFIED CHOLESTEROL (MG. %)	CHOLESTEROL ESTERIFIED (%)	TOTAL CHOLESTEROL (MG. %)	FREE CHOLESTEROL (MG. %)	FREED CHOLESTEROL (%)
1	Fresh	45	4.5	10	50	3.5	7
	Frozen	40	2.5	6.2	55	3	5.4
2	Fresh	52	4.1	7.9	42	4	9.5
	Frozen	46	2.2	4.7	45	2.1	4.7

*After 24 hours of incubation.

RESULTS

Results in both the normal and lymphoma groups were uniform, irrespective of the origin of the tissue (autopsy or surgical). Since our work, however, was done on stored specimens, preliminary experiments were conducted to establish the influence of storage on the cholesterol esterifying and hydrolyzing activity of splenic tissue (Table I). There was significant reduction of activity in frozen as compared to fresh tissue. This, however, was uniform in all samples studied. Thus, our results, although probably inaccurate as to absolute value, seemed significant when similarly preserved normal and pathologic tissues were compared.

Experimental results are presented in detail in Tables II and III. Saline extracts of normal spleens esterified 8 to 15 per cent of cholesterol and

hydrolyzed 5 to 10 per cent cholesterol esters after 24 hours of incubation (Table II). Identical experiments with spleens from lymphoma patients indicated that these extracts also had low esterifying activity but a constantly higher ability

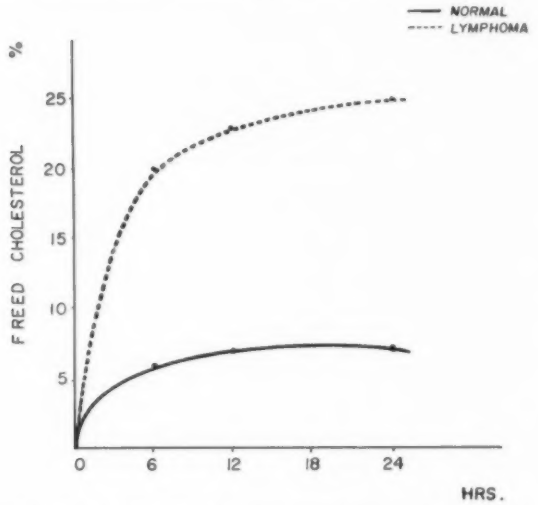

Fig. 1.—Cholesterol ester hydrolysis of normal and lymphoma splenic tissue (calculated on averages of all results).

TABLE II. ESTERIFICATION OF CHOLESTEROL AND HYDROLYSIS OF CHOLESTEROL ESTERS BY EXTRACTS OF NORMAL SPLENIC TISSUE

CASE NO.	TOTAL CHOLESTEROL (MG.)	ESTERIFIED CHOLESTEROL* (MG.)				CHOLESTEROL ESTERIFIED (24 HR.) PER CENT
		0 HR.	6 HR.	12 HR.	24 HR.	
A. Esterification						
1	48	0	4	5	7	14.5
2	55	0	4	4.4	5.2	9.45
3	50	0	4	4.5	5	10
4	45	0	3.2	3.6	4	8.8
5	50	0	4.1	4.5	5.3	10.6
B. Hydrolysis						
CASE NO.	TOTAL CHOLESTEROL (MG.)	FREE CHOLESTEROL* (MG.)				FREED CHOLESTEROL (24 HR.) PER CENT
		0 HR.	6 HR.	12 HR.	24 HR.	
1	50	0	2.5	3	3	6
2	50	0	3	3.5	4	8
3	55	0	2.2	2.6	2.8	5.09
4	50	0	3	5	5	10
5	45	0	4	4	4.5	10

*At different hours of incubation.

to hydrolyze cholesterol esters (Table III and Fig. 1). The partition of cholesterol in the lymphoma spleens studied is presented in Table IV. It would appear that the increase in cholesterol hydrolyzing activity of each sample of lymphoma splenic tissue, although constant, did not bear strict relationship to the degree of decrease of cholesterol esters found in lymphoma tissues.

TABLE III. ESTERIFICATION OF CHOLESTEROL AND HYDROLYSIS OF CHOLESTEROL ESTERS BY EXTRACTS OF LYMPHOMA SPLENIC TISSUE

CASE	TOTAL CHOLESTEROL (MG.)	ESTERIFIED CHOLESTEROL* (MG.)				CHOLESTEROL ESTERIFIED (24 HR.) PER CENT
		0 HR.	6 HR.	12 HR.	24 HR.	
A. Esterification						
1. Hodgkin's sarcoma (♀, 19)	50	0	4	5	6	12
2. Hodgkin's sarcoma (♀, 65)	50	0	3	3.5	5	10
3. Hodgkin's sarcoma (♂, 35)	45	0	4.5	5.4	5.5	12.2
4. Reticulum cell sarcoma (♂, 58)	57	0	5	5.2	5.2	9.1
5. Reticulum cell sarcoma (♀, 22)	49	0	5	5	5	10.2
6. Reticulum cell sarcoma (♀, 62)	54	0	5.2	5.3	5.5	10.1

B. Hydrolysis

CASE	TOTAL CHOLESTEROL (MG.)	FREE CHOLESTEROL† (MG.)				FREED CHOLESTEROL (24 HR.) PER CENT
		0 HR.	6 HR.	12 HR.	24 HR.	
1	50	0	6.5	8	10	20
2	45	0	6.5	8	8	18.2
3	53	0	12	12	13.2	24.9
4	55	0	8	10	11	20
5	47	0	10	10	10.3	21.9
6	50	0	8.5	9	10	20

*At different times of incubation.

†The whole amount of cholesterol in the mixture was preliminarily esterified. Attempts to determine chemically free cholesterol in the solution by the method of Schoenheimer and Sperry, after the esterification, gave negative results.

TABLE IV. CHOLESTEROL IN LYMPHOMA SPLEENS

CASE*	TOTAL (MG. %)	FREE (MG. %)	ESTERIFIED (%)
1	580	208	64.1
2	670	490	26.8
3	648	240	63.0
4	420	165	60.7
5	615	275	55.3
6	400	152	62.0

*Numbers correspond to those given in Table III, *A*.

DISCUSSION

Mueller[7] first showed that cholesterol esters could be synthesized from free cholesterol in incubation with tissue extracts. The hydrolysis of cholesterol esters by extracts of liver tissue was first demonstrated by Schultz.[8] Enough evidence is available today to conclude that continuous mechanisms of synthesis and hydrolysis of cholesterol esters are operative in the body under the influence of tissue esterases.[9] Swell and associates[3] reported the presence of enzymatic activity in pancreas, intestinal mucosa, liver, and kidney of the rat and suggest a possible role of cholesterol esterase in the synthesis and hydrolysis of tissue and blood cholesterol esters. Hydrolytic cholesterol esterase activity has been found in liver, pancreas, intestinal and gastric mucosa, spleen, kidney, muscle of dog, swine, horse, and rabbit.[10] Two hydrolytic enzymatic systems are probably present in animal tissues.[11] One (found in liver, kidney, spleen, lung, testes, and intestinal mucosa) acts optimally at pH 5.3; another (found only in pancreas) has optimal activity at pH 7.0. Our studies tried to point out the possible presence of an hydrolyzing cholesterol esters system in human splenic tissue and indicated the significance of its variations in pathologic conditions.

The present study also clarified at least in part, the mechanism of accumulation of free cholesterol in the splenic tissue of patients with lymphoma. The following possible explanations presented themselves when the phenomenon was first observed: (1) poor activity of cholesterol esterifying system in lymphoma tissues, with consequent poor synthesis of cholesterol esters; (2) high activity of cholesterol esters hydrolyzing system in these tissues; (3) possible bondage of more free cholesterol by proteins in the cells of lymphoma spleens. On the basis of our results the second explanation appeared more reliable. We previously described that the changes in cholesterol distribution typical of lymphoma may be found, to a lesser extent, in diseases where there is involvement of the spleen, irrespective of its nature. Preliminary results showed similarly, although less strikingly, increased activity of hydrolyzing cholesterol system in these tissues as well. Thus, the phenomenon described might be of general significance, although, perhaps, of greatest magnitude in lymphoma.

SUMMARY

1. Normal splenic tissue extracts show poor esterifying and hydrolyzing activity of cholesterol. Activity decreases further on storage of tissue, even at freezing temperature.

2. Extracts of splenic tissue from patients with lymphoma also possess rather poor cholesterol esterifying activity, while exhibiting considerably higher cholesterol esters hydrolytic activity.

3. These findings contribute to the explanation of the low degree of esterification of cholesterol in tissue of patients with lymphoma.

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THE EFFECT OF CO_2 INHALATION ON THE CONJUNCTIVAL VESSELS, BLOOD pH, AND pCO_2 IN YOUNG DIABETIC SUBJECTS

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CARDIOVASCULAR-RENAL disease has replaced nearly all other causes of death in juvenile diabetes and accounts for 87.5 per cent of the total. Nephropathy alone is responsible for one-half of the deaths. By the age of 30, 63 per cent of those patients with juvenile diabetes surviving 20 years of their disease have retinopathy and 29 per cent have retinitis proliferans.[1]

The first sign of retinopathy in juvenile diabetic subjects is venous congestion.[2, 3] The retinal venular-capillary microaneurysms are considered to be secondary to prolonged venous stasis.[4] By viewing the conjunctival vessels microscopically, venular dilation has been found to precede the retinal changes.[5] The venular engorgement is particularly pronounced in young patients with poorly controlled diabetes, with or without clinical keto-acidosis.[5] Pregnancy, which tends to produce metabolic acidosis and to be accompanied by a fall in pCO_2,[6, 7] also aggravates this conjunctival vascular response.[8] Diabetic retinopathy has been found in some cases to be produced or increased during pregnancy and to decrease or disappear after delivery.[9, 10]

The effect of changes in blood oxygen tension in the human has been well exemplified in studies on retrolental fibroplasia. Abnormal changes in blood oxygen tension in the newborn infant have been found to produce severe arteriolar constriction and venular distension in the retina.[11-13] This sometimes has become irreversible and has been accompanied by retinal hemorrhages, proliferation of new retinal vessels and blindness.

In the absence of evidence for changes in blood and tissue oxygen tension, it must not be forgotten that CO_2 also is notorious for its effects on the small blood vessels.[14] It has been remarked that tissue reactions to different stimuli are commonly stereotyped. Thus, the cumulative effects of innumerable variations in local tissue pCO_2 may over many years stimulate, in the much slower-reacting vessels and tissues of the adult, a similar kind of process in diabetics of retinitis proliferans. In support of this working hypothesis[15] are the well-known variations in acid-base balance which occur in diabetes. The Henderson-Hasselbalch equation (referred to later) relates changes of a metabolic nature, i.e., involving the bicarbonate, to variations in pCO_2. In the blood,

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pCO_2 levels are supposedly maintained at a fairly constant level by the respiratory center and respiratory ventilation.[16, 17] However, during diabetic acidosis and coma the blood pCO_2 usually is abnormally low,[6] and Lundbaek has found that even in *nondiabetic* patients the alveolar pCO_2 varies with insulin administration and carbohydrate consumption.[18, 19] Therefore, in so-called well-controlled diabetics, even greater pCO_2 fluctuations may be anticipated. Also, local peripheral vascular stasis and possibly other factors may impede the dissipation of the usually rapidly diffusible tissue CO_2 into the blood. An additional factor is that cell membranes are more permeable to CO_2 than other acids with the result that the intracellular pH is rapidly lowered by the slightest increase in local pCO_2.[16] Consequently, it is possible that either tissue acidosis or variations in the CO_2 tension causes venular dilation, the apparent precursor of retinopathy, in diabetic subjects.

Thus the purpose of this investigation was to observe the effect on the conjunctival vessels of increases in tissue and blood pCO_2 and acidosis in young diabetics.

PLAN OF STUDY

"BASAL" EXAMINATION : Blood pH, pCO_2 and plasma bicarbonate content; conjunctival vessel observation and photography.

INHALATION PERIOD : 30 minutes of 5 per cent carbon dioxide.

CARBON DIOXIDE EQUILIBRIUM EXAMINATION* : Blood pH, pCO_2 and plasma carbon dioxide content; conjunctival vessel observation and photography.

* *Beginning 25 minutes after started inhalation.*

Fig. 1.

MATERIAL AND METHODS

All the subjects examined were diabetics with and without retinopathy and nephropathy, but clinically healthy in other respects. The only selection made was that of age, i.e., all subjects were less than 33 years old. The subjects were divided into 2 categories. The first group was made up of 11 hospitalized subjects in whom the blood gas determinations were made on *arterial* blood. The second group (9 experiments in 5 cases of long-term diabetics) was made up of ambulatory patients in whom the blood gas determinations were made on *venous* blood.

Fig. 1 surveys the plan of study. Preinhalation examination included the determination of blood pH, plasma bicarbonate content, and therefrom the CO_2 tension determination, as well as observation and photography of the conjunctival vessels. The blood for the gas determinations was obtained anaerobically, either by arterial puncture or venipuncture. In the case of arterial puncture, a few milliliters of 2 per cent procaine were injected deeply about the brachial artery just above one elbow, and after 3 minutes a sterile heparin-washed Cournand needle was inserted into the artery. The needle was kept in place during the whole period of study. After 3 or more minutes, 10 to 20 ml. blood were withdrawn into heparinized syringes. If venous blood was used for gas determinations, the subjects were placed at absolute rest for 20 minutes before the blood samples were taken.

Thereafter the conjunctival vessels were observed and photographed as described previously.[20] A mask with inflated rim was then carefully adjusted to the contour of the patient's face to effect an airseal. The slightest leak during the course of many preliminary studies with two inferior face-masks coincided with unincreased pCO_2's and even, surprisingly enough, occasionally in decreases of up to 10 mm. in venous pCO_2. The subject then inhaled the 5.0 per cent (±0.25 per cent) CO_2 in air for 30 to 35 minutes. On one occasion 7.5 per cent CO_2 was given for 20 minutes but the discomfort was such that with the next subject the use of this concentration was discontinued after 12 minutes' administration. The inhalation was continued for 30 minutes in order to reach equilibrium with the vessel walls and perivascular tissue. Then another specimen of blood was drawn and the same field of the conjunctival vessels was photographed. The subjects were observed for a few minutes after inhalation for any possible aftershock, as described by Alexander and associates.[21] In 47 studies, shock was encountered in only 2 cases (not included in the present study), in which pallor, shivering, and sweating were displayed in the postinhalation period. However, these symptoms responded well to sweet drinks and thus appeared clinically to be mild insulin reactions.

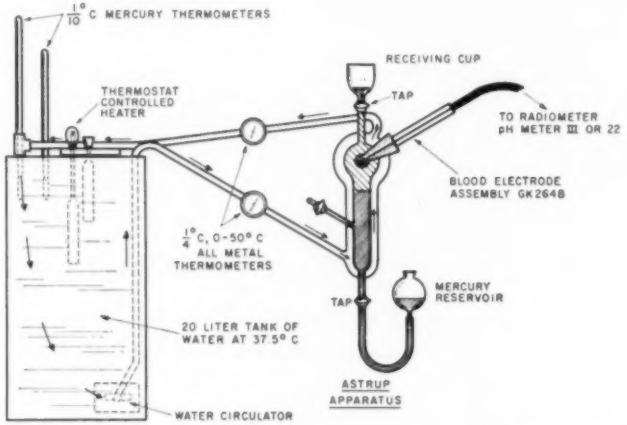

Fig. 2.—Diagram of Astrup apparatus, electrode assembly, and thermostatic water circulation.

The blood specimen (usually immediately, and always within 3 minutes) was introduced beneath mercury into the chamber of an Astrup apparatus (Fig. 2) for measurement of the pH. The Astrup method[22, 23] was used with the following modifications. The temperature of the thermostat-controlled water tank was maintained at 37.5° C. ±0.25° C. Two mercury thermometers were present in the 20-liter thermostatically controlled water tank. Weston all-metal thermometers were placed at the inlet and outlet of the water jacket of the Astrup chamber.

The standardization of the pH meters was, as far as applicable, according to the procedures annotated,[22-26] and with U.S. National Bureau of Standards (N.B.S.) buffers. The N.B.S. standard buffer values used were interpolated for 37.5° C. from those supplied, as:

Borax	9.083 pH (N.B.S.) units[27, 28a]
Equimolar solution of potassium Dihydrogen phosphate and disodium Hydrogen phosphate	6.825 pH (N.B.S.) units[28b]
Potassium hydrogen phthalate	4.025 pH (N.B.S.) units[28c]

The pH 3 and pH 22 meters* were used in the present investigation. The Beckman PhG meter was used only in preliminary studies, not reported here, as it was found inadequate for the order of accuracy required. The pH meter in use was calibrated against at least two of the buffer standards both before and after each study. The pH meter was also checked for zero drift between the two blood pH readings and immediately at the end of each study. Correction for glycolysis was made from duplicate readings at timed intervals. The potentiometer scale calibrations of the pH meter were checked and found accurate against a standard potentiometer.†

As soon as the erythrocytes had settled sufficiently, determination of the bicarbonate content was made on uncentrifuged plasma (to avoid the centripetal displacement of CO_2 into the overlying mineral oil during centrifugation) according to the method of Peters and Van Slyke[29] using 1 ml. and 0.5 ml. Van Slyke pipettes corrected for plasma delivery.[26] The "C" correction factor was determined using a blank sample of 1 ml. or 0.5 ml. of CO_2-free water. The determination of the plasma CO_2 content was repeated until the results of two or more determinations were within at least ±0.5 per cent.

Calculations of the pCO_2 were made according to the Henderson-Hasselbalch equation rearranged:

$$pCO_2 = CO_2 \text{ content}/(S\ 10^{pH} - pK^1 + 1)$$

where "S" is the solubility factor for CO_2 estimated to be 0.0304 at 37.5° C.[26] The pK^1 was assumed to be 6.09.[30]

RESULTS

Plasma Bicarbonate Content.—In the 11 arterial blood studies (Table I), the preinhalation arterial plasma bicarbonate content averaged 24.84 mEq./L. The normal range quoted is from 18.5 to 24.7.[6] Six of the diabetic patients had values for arterial plasma bicarbonate content above this range. After inhalation, the plasma bicarbonate content was increased in 7 out of 11 arterial studies from 1.5 to 3.3 mEq./L. (6 to 14 per cent). The average increase was 1.63 mEq./L. or 6.5 per cent. No significant change was apparent in the venous bicarbonate content studies (normal range 26 to 33 mEq./L.[17]) (Table II). Therefore, it may be deduced that passage of the blood through the peripheral tissues eliminates the effect of the inhalation of 5 per cent CO_2 on the plasma bicarbonate content.

pH in Blood.—The pH in arterial blood, before inhalation, averaged 7.417 (Table I) and in venous blood 7.385 (Table II). The values for normal people are taken to be 7.38 to 7.43 for arterial blood,[31] and 7.35 to 7.45 for venous blood.[17] The hydrogen ion concentration decreased during the 30-minute inhalation on the average of 0.089 pH units in arterial blood (Table I) and 0.035 pH units in venous blood (Table II). In 4 patients the arterial blood pH decreased 0.104 to 0.161 pH units. The greatest change in pH, i.e., the poorest ability to buffer the respiratory acidosis, occurred in the 4 worst of the 5 retinopathy cases.

The preinhalation arterial pH appeared acid in 3 and alkaline in 4. The preinhalation venous pH in only one study was found to be on the acid side of normal. Thus there *appeared* to be a wide variation in pH, and therefore in capacity to buffer, in the arterial blood of patients with diabetes.

*Radiometer Corporation, Copenhagen, Denmark.

†Courtesy of Professor J. J. Lingane, Coolidge Laboratory, Harvard University, Boston, Mass.

TABLE I. PERIPHERAL VASCULAR (CONJUNCTIVAL) AND ARTERIAL BLOOD

NO.	SEX	AGE	DURATION OF DIABETES (YR.)	INSULIN DOSE		HOURS BEFORE STUDY	COMPLICATIONS		PLASMA BICARBONATE CONTENT (mEq./L.)		
				CRYSTALLINE	NPH		RETINOPATHY	NEPHROPATHY	INHALATION AIR	INHALATION 30 MIN. 5% CO_2	INCREASE
A1	M	22	1.5	10	30	5.5	-	-	26.92	28.43	1.51
A2	M	18	3.0	16	52	4.5	-	-	25.47	26.41	0.94
A3	M	19	13.0	4	68	7.0	-	-	25.70	25.40	-0.30
A4	M	29	6.0	0	36	8.5	-	-	23.88	27.66	3.28
A5	F	23	14.0	6	44	4.3	+	-	20.37	22.45	2.08
A6	M	30	20.0	0	38	4.0	+	+	28.30	30.51	2.21
A7	F	28	14.0	6	38	6.5	+	+	22.46	25.39	2.93
A8	M	25	0.2	0	24	7.5	-	-	24.10	26.41	2.31
A9	M	20	9.0	6	28	2.5	-	-	25.91	27.75	1.84
A10	F	25	15.0	8	84	5.0	+	-	24.13	25.06	0.93
A11	M	24	15.0	8	66	7.5	+	+	26.01	26.17	0.16
Average		24	13.7		52	5.7			24.84	26.47	1.63
Normal Ranges:									18.5-24.75		

*Assuming pK = 6.09.[26] Figures rounded to nearest decimal place.

TABLE II. PERIPHERAL VASCULAR (CONJUNCTIVAL) AND VENOUS BLOOD

NO.	SEX	AGE	DURATION OF DIABETES (YR.)	INSULIN DOSE		HOURS BEFORE STUDY	COMPLICATIONS		PLASMA BICARBONATE CONTENT mEq./L.		
				CRYSTALLINE	NPH		RETINOPATHY	NEPHROPATHY	INHALATION AIR	INHALATION 30 MIN. 5% CO_2	DECREASE
V1	F	32	18	6	36	3.0	+	+	27.58	27.18	0.40
V2	F	29	16	4	50	7.0	+	+	25.02	26.42	+1.40
V3a	F	31	27	6	30	3.2	+	-	29.52	28.55	0.97
b						2.5			26.19	26.19	0.00
V4a	F	23	20	-	48	6.3	+	+	26.16	25.04	1.12
b						4.5			23.10	23.67	+0.57
V5a	M	30	20	-	38	7.5	+	+	32.38	32.53	+0.15
b						6.0			31.18	30.88	0.30
c						3.25			30.59	30.19	0.40
Average		29	20.2		44	4.8			27.97	27.85	0.12
Normal Ranges									26-33		

*Assessing pK = 6.09.[26] Figures rounded to nearest decimal place.

STUDIES DURING 5 PER CENT CO_2 INHALATION (30 MIN.) IN DIABETIC SUBJECTS

RELATIVE pH VALUES			pCO_2 (mm.Hg)*			CONJUNCTIVAL VESSELS				
INHALATION		ABSOLUTE DECREASE	INHALATION		INCREASE	VASCULAR PATTERN		RESP. TO INHAL.		ERYTHROCYTE AGGREGATION‡
AIR	30 MIN. 5% CO_2		AIR	30 MIN. 5% CO_2		BEFORE	AFTER	ART. CONSTRICTION†	VEN. DILATION†	
7.317	7.302	0.015	49.6	53.7	4.1	N	I	0	1	1
7.286	7.223	0.063	50.2	59.6	9.4	I	I	0	0	1
7.473	7.400	0.073	33.6	39.0	5.4	I	I	0	1	3
7.510	7.431	0.079	28.8	39.0	10.2	N	I	1	2	0
7.464	7.315	0.149	26.9	42.4	15.5	I	I	0	2	3
7.452	7.346	0.106	39.6	53.7	14.1	I	I	0	1	3
7.478	7.317	0.161	29.1	46.7	17.7	II	II	0	0	3
7.416	7.318	0.098	35.8	48.5	12.7	N	I	0	2	1
7.480	7.414	0.066	33.9	41.3	7.4	N	I	0	2	1
7.329	7.225	0.104	43.5	56.2	12.8	N	N	0	0	1
7.361	7.293	0.068	43.5	51.9	8.4	I	I	0	1	3
7.415	7.326	0.089	37.7	48.4	10.7					
7.38-7.43			35-43			N		0	0	0

†Graded 0 to 2. ‡Graded 0 to 3.+

STUDIES DURING 5 PER CENT CO_2 INHALATION (30 MIN.) IN DIABETIC SUBJECTS

RELATIVE pH VALUES			pCO_2 (mm.Hg)*			CONJUNCTIVAL VESSELS				
INHALATION		ABSOLUTE DECREASE	INHALATION		INCREASE	VASCULAR PATTERN		RESP. TO INHAL.		ERYTHROCYTE AGGREGATION‡
AIR	30 MIN. 5% CO_2		AIR	30 MIN. 5% CO_2		BEFORE	AFTER	ART. CONSTRICTION†	VEN. DILATION†	
7.443	7.369	0.074	38.5	44.7	6.2	II	II	0	0	3
7.422	7.380	0.042	36.6	42.4	5.8	II	II	0	0	3
7.376	7.338	0.038	47.8	50.2	2.4	I	I	0	1	2
7.389	7.338	0.051	41.2	46.1	4.9	I	I	0	1	3
7.383	7.296	0.087	41.7	48.3	6.6	II	II	0	0	3
7.354	7.283	0.071	39.2	46.9	7.7	II	II	0	0	3
7.328	7.296	0.032	58.2	62.7	4.5	I	I	0	1	3
7.396	7.410	+0.014	49.3	46.4	−2.9	I	I	0	0	3
7.374	7.436	+0.062	49.7	42.9	−6.8	I	I	1	1	3
7.385	7.350	0.035	46.9	47.8	3.2					
7.35-7.45			41-47			N		0	0	0

†Graded 0 to 2. ‡Graded 0 to 3.+

Carbon Dioxide Tension.—Preinhalation CO_2 tensions in arterial blood (normals 35 to 43 mm. Hg[6, 31]) appeared abnormally low in 5 and abnormally high in 2 diabetic subjects (Table I). Of the venous study (normals 41 to 47 mm. Hg[6]), 3 had low pCO_2's and one had persistently abnormally high pCO_2's.

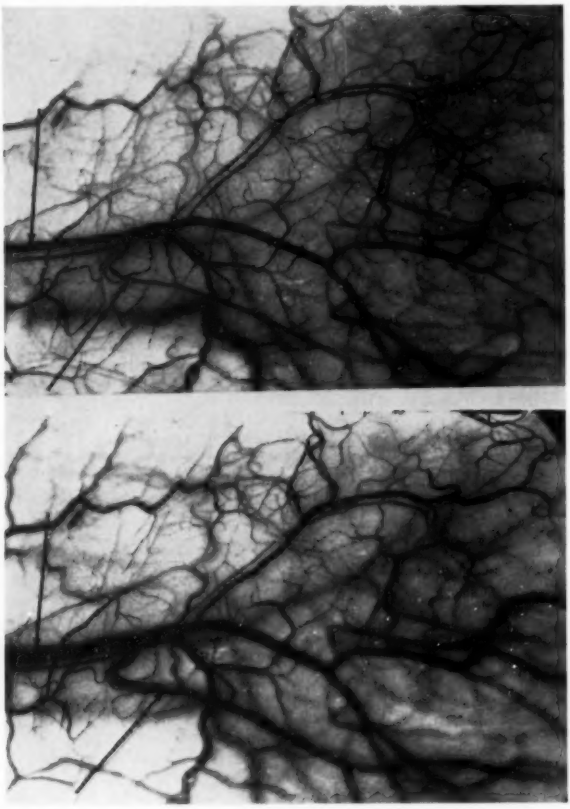

Fig. 3.—Venular dilatation in response to inhalation of 5 per cent carbon dioxide (Case A8).

This latter case (V5, Table II) had a normal arterial pCO_2 (A6, Table I). His venous pCO_2, estimated on 6 occasions during a period of 4 months, ranged between 51 and 60 mm. Hg. Thus, the venous pCO_2 was 11 to 20 mm. higher than the arterial pCO_2 estimation. His venous blood pH on these occasions was always low, 7.35 or below, although the plasma bicarbonate content, on the contrary, was always high normal.

Whereas, in general, venous pCO_2 was only slightly increased (3 to 4 mm. Hg) by the inhalation (Table II) the arterial pCO_2 increased an average of 10.7 mm. Hg (range 4 to 18 mm.) (Table I). The arterial blood biochemical studies were considered more representative of the effect of the inhalation on the peripheral vessels and the tissues than the venous studies.

Conjunctival Vascular Changes.—In the preinhalation studies the patients showed 3 distinguishable vascular patterns divided according to the caliber of the arterioles and venules. Normal vascular pattern (A-V ratio 1 : 3 to 1 : 2) was found in 5 cases; vascular pattern-change I (A-V ratio 1 : 8 to 1 : 4) was found in 10. Vascular pattern-change II (A-V ratio 1 : 1) with marked arteriolar constriction and tissue ischemia, as indicated by capillary closure, was found in another 5 cases.[8, 33]

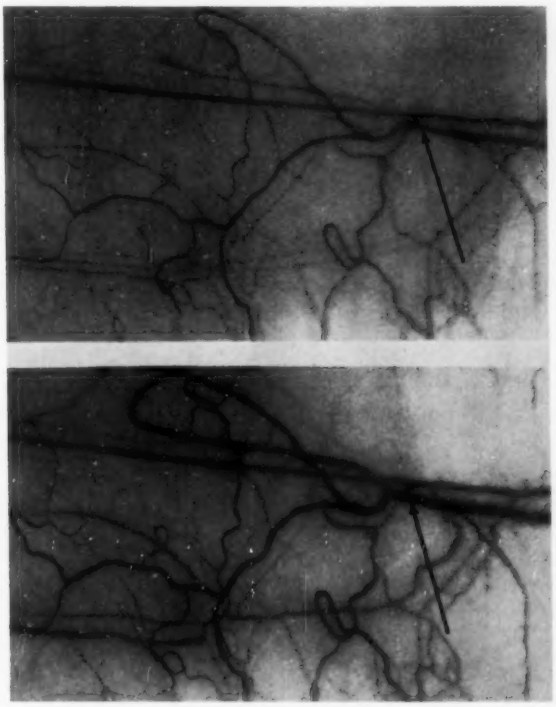

Fig. 4.—Venular dilatation in response to inhalation of 5 per cent carbon dioxide (Case A4).

After CO_2 inhalation the most significant change in the smaller blood vessels was a generalized, nonuniform, congestion and dilation of the venules which occurred in 12 out of the 20 studies. In 4 of these cases, venular dilation was marked (Grade 2) (Figs. 3 and 4). Three of these latter cases were accompanied by marked slowing of erythrocyte velocity and perivenular edema formation. In most respects these 4 patients showed more than the average degree of biochemical changes. Thus, their bicarbonate content increases were 1.8, 2.0, 2.3, 3.3 mEq./L.; pH changes of 0.066, 0.079, 0.098, 0.149; and pCO_2 increases of 7.4, 10.2, 12.7, 15.5 mm. Hg, compared with average changes of 1.6 mEq./L., 0.089 pH unit and 10.7 mm. Hg, respectively.

All cases showing vascular pattern-change II had extensive smaller blood vessel disease (retinitis proliferans and nephropathy), and in none of those did

CO_2 inhalation produce any vascular change despite maximal pCO_2 increases (arterial, 17.7; venous, 6.2, 5.8, 6.6, 7.7 mm. Hg). All the subjects who did respond showed either normal vascular patterns or vascular pattern-change I.

No definite change was discernible in the arterioles in 18 out of 20 cases. However, in 2 cases there was a definite slight arteriolar constriction. In one of these patients (Table II, V5c), arteriolar constriction was only observed once out of 4 studies.

The degree of intravascular erythrocyte aggregation was unchanged during the inhalation period.

DISCUSSION

These experiments demonstrate that the respiratory acidosis produced by inhalation of 5 per cent CO_2, directly or indirectly, causes conjunctival venular engorgement in young diabetics. The vascular response is uniform in type although it differs quantitatively from subject to subject. Bloch,[32] in describing microscopic observations of the circulating blood in the bulbar conjunctiva during disease and health, stated that slight dilatation of the walls of small venules (measuring 20 to 50 μ) occurred whenever the majority of the erythrocytes were in aggregates which measured more than 12 μ in diameter and the linear velocity of flow was reduced. He found this venular dilatation in lung diseases such as lobar pneumonia and active tuberculosis. Although extensive intravascular erythrocyte aggregation, by producing stasis with concomitant anoxia and carbonic acid accumulation, might alone produce venular dilatation, it is evident that the above diseases are likely to be associated also with primary pCO_2 changes. In a study of the conjunctival vessels in diabetic children, a positive relationship has also been found between the degree of erythrocyte aggregation in arterioles, capillaries, and venules, and the degree of venular distension.[33] However, this relationship is not always present, i.e., venular distension can be found in cases showing no aggregation, while in cases with marked erythrocyte aggregation no venular distension may be present. These phenomena in diabetes mellitus (erythrocyte aggregation and venular distension) are not necessarily dependent on each other. This view is also supported from the data of the present experiments. During these studies no change in the degree of intravascular erythrocyte aggregation was noted, although a pronounced venular dilatation occurred. It appears that either acidosis or changes in the CO_2 tension, exaggerated by local factors, cause venular dilatation, perivenular edema, and occasionally arteriolar constriction. Perhaps the preservation of normal vascular function is dependent upon optimum pCO_2 and pO_2 tissue : blood equilibrium and sudden fluctuations in these equilibriums may result in the same stereotyped vascular and perivascular tissue changes.

In the present study of diabetics on which arterial blood was taken, the data reveal lack of uniformity in the preinhalation levels of plasma bicarbonate (20 to 28 mEq./L.), blood pH (7.286 to 7.510) and pCO_2 (29 to 50 mm. Hg). This wide range of results in hospitalized young diabetic patients might be explained by the finding by Lundbaek[18] of altered respiratory-center sensitivity

in subjects receiving insulin. It must be borne in mind that glass electrodes frequently have different characteristics and give different simultaneous pH readings on the same blood samples. Comparing Beckman and Radiometer electrode assemblies (and well calibrated pH meters) it was found, however, that despite the difference in simultaneous "absolute" pH readings the differences in pH obtained by each electrode, before and after CO_2 inhalation, were very much the same.

The patients with retinopathy were found to resist poorly the effect of respiratory acidosis, i.e., to show the largest pCO_2 changes (Table I, Cases 5 to 7, 10, and 11). Likewise, the cases which did not exhibit vascular responses and were found to have poor ability to minimize the effect of CO_2 inhalation were those diabetics with irreversible peripheral vascular changes in the conjunctivae (vascular pattern-change II), in the retinae (retinitis proliferans) and most probably in the kidneys (casts, albuminuria, delayed phenolsulfonphthalein excretions, etc.).

This indicates the possibility of a causal relationship between the disturbance in acid-base metabolism, more specifically poor buffering ability, and the vascular disease affecting the retina, kidneys, and conjunctiva in diabetes mellitus.

SUMMARY

Inhalations of 5 per cent CO_2 for 30 minutes by young diabetic patients produced venular dilation, slowing of erythrocyte velocity, occasional perivenular edema and arteriolar constriction, average arterial blood pH decreases of 0.089 pH units, average plasma bicarbonate content increases of 1.63 mEq./L. and average arterial pCO_2 increases of 11 mm. Hg. The most marked pCO_2 and pH changes occurred in the diabetic subjects with the most degeneration of the small blood vessels.

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EFFECTS OF INTRAVENOUS HUMAN PLASMIN ON THE BLOOD CLOTTING MECHANISM OF THE DOG

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PLASMIN (fibrinolysin) is an active proteolytic enzyme which is normally present in human and animal plasma as its precursor plasminogen (profibrinolysin). Its physiologic function has not been completely elucidated; but it is thought to play a major role in the dissolution of hemostatic fibrin clots, and it has been proposed as the factor responsible for the fluidity of menstrual blood which is devoid of fibrinogen and low in other clotting components.[1] Excessive activation of plasminogen to plasmin has been incriminated as the etiologic factor in a hemorrhagic diathesis seen in some postsurgical[2] and obstetrical patients[3] and in certain patients with prostatic carcinoma.[4]

There is some experimental evidence to suggest that an infusion of plasmin might be of value in the proteolytic dissolution of intravascular thrombi of recent origin.[5] However, since, at the same time, plasmin has been indicted as an agent capable of producing a diminution in clotting factors of a magnitude sufficient to induce a generalized hemorrhagic diathesis, considerable care is necessary in the therapeutic use of this enzyme.

Although some studies have been reported concerning alterations in fibrinogen following plasmin infusion in the dog,[5] we can find no reports of any attempt to study other clotting factors. It has been the purpose of this investigation to collect data concerning alterations in fibrinogen, fibrinolytic activity, prothrombin, factor V, factor VII, and thromboplastin generation after a constant infusion of plasmin in varying dosages to large healthy dogs. The objectives have been twofold: (1) to find the optimal time-dosage regimen capable of producing maximal fibrinolytic activity with minimal alteration in other clotting factors; and (2) to delineate the changes in the coagulation mechanism produced by an excess of circulating plasmin.

METHODS

The human plasmin* used in this study was plasminogen, obtained through further purification of Cohn's fraction III, which was activated with streptokinase, ultraviolet-irradiated, and lyophilized. The streptokinase used independently in several experiments was the same preparation used in the activation of the plasminogen preparation. The

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plasmin was supplied in vials containing 28,000 Christiansen units per vial, as assayed by the manufacturer. In each instance, the dosage selected was administered by constant intravenous infusion in 500 c.c. of 5 per cent dextrose in water over a 2-hour period.

Blood samples were drawn in siliconed syringes from an exposed femoral vein prior to the infusion, at 30-minute intervals during the infusion, and 1 and 2 or 3 hours post-infusion. The blood was collected in calibrated siliconed test tubes containing a standard volume of 1.85 per cent potassium oxalate. The methods used for fibrinogen,[6] fibrinolysis,[7] prothrombin,[8] factor V,[9] factor VII,[10] and thromboplastin generation[11] have been previously described. Periodic rectal temperatures were taken during the course of the infusion. The femoral cut-down wound was observed for evidences of abnormal bleeding.

Infusions of human plasmin (with some of the originally added streptokinase present) were administered to dogs varying in weight from 23 to 30 Kg., the usual schedules being 1,900, 3,000, 3,700, 6,500, and 9,000 units per kilogram of body weight. Since the original plasminogen preparation had been activated with 100,000 units of streptokinase per vial and considerable residual streptokinase activity was demonstrated in the final lyophilized preparation, several dogs were given infusions of 250,000 units of the streptokinase preparation alone to compare the proteolytic changes with those produced by plasmin administration. Since hydrocortisone has been used empirically as a therapeutic antagonist in patients with a hemorrhagic diathesis of fibrinolytic origin, 3 dogs were given separate but simultaneous infusions of 6,500 or 9,000 units of plasmin per kilogram of body weight and 50 to 100 mg. of hydrocortisone sodium succinate over the same 2-hour period.

RESULTS

Infusion of plasmin in a dosage of 1,900 units/Kg. body weight produced no change in circulating fibrinogen; one blood sample during the infusion period demonstrated moderate fibrinolytic activity. At a dosage of 3,000 to 3,700 units/Kg. body weight the fibrinogen progressively fell over the 2-hour infusion period from a normal of 355 mg. per cent to nonmeasurable levels at the end of the infusion. After the first half hour of infusion fibrinolytic activity was marked and remained so throughout the period of observation; total proteolytic degradation of a fibrin clot occurred in all samples taken at one hour and thereafter. Dosages in this range resulted in slight decreases in factor V and factor VII activity; the 3,700 unit schedule produced marked prolongation of thromboplastin generation (to the range of 10 per cent of normal when plotted against dilutions of normal plasmas).

Infusions of 6,500 to 9,000 units per Kg. body weight produced total loss of circulating fibrinogen within one-half to one hour after the start of the infusion; proteolytic activity of the plasma was marked, producing total disintegration of fibrin clots even at the first half-hour sample. Marked decreases in factor V as well as in thromboplastin generation occurred. There was a slight decrease in factor VII activity to that of 50 per cent plasma. A minimal prolongation in one-stage prothrombin time could be explained on the basis of factor V depletion alone. Fig. 1 shows the changes produced by the infusion of 6,500 Christiansen units/Kg. body weight to a dog weighing 30 Kg.

Simultaneous infusion of hydrocortisone produced no direct inhibition of the proteolytic effects of plasmin.

Infusions of 250,000 units of streptokinase resulted in marked total fibrinolysis without any appreciable change in circulating fibrinogen or in any of the other clotting factors.

Repeated infusions of plasmin to the same dog resulted in gradually increasing tolerance to the effects of plasmin. One dog received 6 infusions of plasmin over a period of 6 months. The last two infusions of 6,500 units per Kg. body weight resulted in considerable plasma fibrinolytic activity but no change in fibrinogen or any of the other clotting factors on either occasion. It is quite possible that the fibrinolytic activity resulted from the presence of residual streptokinase in the preparation rather than to any effect of plasmin itself.

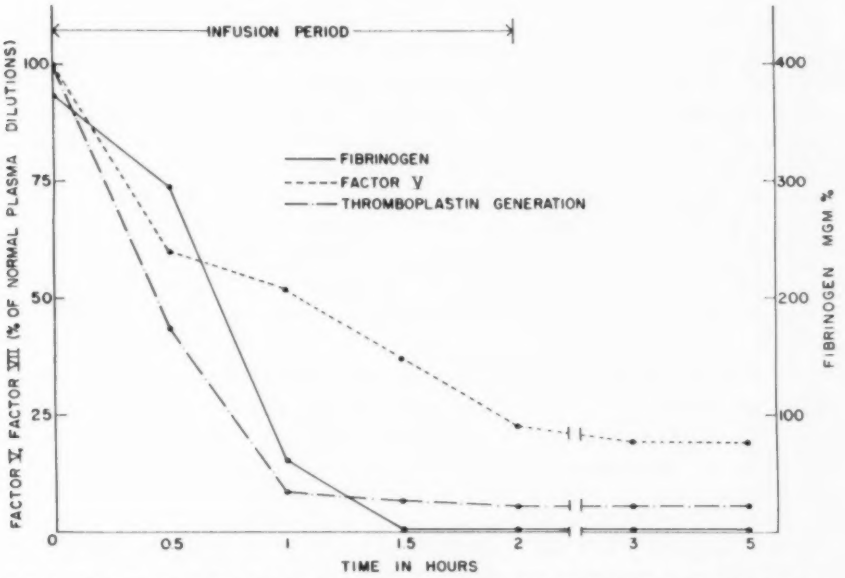

Fig. 1.—Infusion of a dog weighing 30 kilograms with 6,500 units of plasmin per kilogram of body weight.

DISCUSSION

Knowledge concerning alterations in the clotting mechanism produced by an intravenous infusion of human plasmin is of value from several aspects. More information concerning the depletion of specific clotting factors in the hemorrhagic diathesis which follows excessive spontaneous activation of the plasminogen-plasmin system will be of value in the rational treatment of patients with this bleeding abnormality. From another point of view, the deliberate use of plasmin as an agent for producing dissolution of intravascular thrombi requires a knowledge of time-dosage relationships and of the danger of hemorrhage from too rapid administration or excessive dosage of this agent.

These studies suggest that plasmin may affect all 3 phases of the clotting mechanism: thromboplastin generation, prothrombin activation, and fibrin formation. Certainly the major action of plasmin centers around its proteolytic

effects on fibrin and fibrinogen, but massive amounts appear to have an influence on generation of thromboplastin. One cannot definitely assume that changes measured in the thromboplastin generation test are in fact due to changes in the first stage of clotting, since alterations in factor VII may also influence this test. The fall in factor VII observed in our in vivo studies is known to occur in vitro. From the standpoint of treatment of a hemorrhagic state secondary to excessive circulating plasmin, replacement of the substrate defect could be accomplished by administration of fresh plasma or whole blood, preferably supplemented with large amounts of lyophilized fibrinogen. Although we have not been able as yet to localize a specific defect in the first phase of the clotting mechanism, the abnormality may be a deficiency of antihemophilic globulin; since the Michigan Department of Health has a lyophilized preparation of antihemophilic factor, we would also be inclined to use this agent as well in the treatment of a hemorrhagic diathesis of this nature. This is a passive form of treatment, however, which supplies depleted substrate but has no direct effect on inhibition of plasmin effects; it would be far more desirable to induce a direct inhibition of plasmin. Under the conditions of this experiment, hydrocortisone was ineffective in spite of the apparent improvement noted by Stefanini[12] and by Coon and Hodgson[13] in several clinical cases. We plan to study in vivo inhibition of plasmin with a platelet factor reported to be effective in vitro.

Under the conditions established for this study, satisfactory fibrinolytic effects were not observed until the totally administered dosage of plasmin was such that marked decreases in fibrinogen level and variable alterations in thromboplastin generation also occurred; optimal fibrinolysis was obtained with a minimum dosage of plasmin of 3,000 to 4,000 units/Kg. body weight over the 2-hour infusion period. Streptokinase alone was capable of producing almost complete fibrinolysis with negligible changes in any of the other clotting factors. The difference in results following the injection of streptokinase alone may be explained by the fact that, in the absence of human plasminogen, activation of the dog's own plasminogen occurs to a lesser degree than when streptokinase and small amounts of human plasminogen are administered together.[14, 15]

It is quite possible that plasmin could be used effectively in smaller doses on a regional basis; for example, for distal injection into a popliteal artery after operative removal of an embolus in order to lyse clot propagating distal to the site of embolus. Under these conditions local concentration would be maximal with considerably less of a systemic effect.

Since pyrogenic response was minimal in these animals and other observers have shown that this agent has some effect on the dissolution of recent thrombi, we feel that plasmin could be used safely in humans. We are planning a cautious approach with relatively small dosages in the treatment of recent superficial venous thrombi where an objective evaluation can be made relative to the disappearance of the thrombotic process. It is essential that these patients be followed as well with repeated studies of changes in the clotting mechanism.

SUMMARY

These studies suggest that plasmin in sufficient concentration may alter all 3 phases of the clotting mechanism in vivo. These changes are discussed with respect to the clotting abnormality associated with excessive fibrinolysis seen in some patients with a hemorrhagic diathesis.

Satisfactory time-dosage infusion data for cautious administration of plasmin to humans have been presented. If satisfactory proteolytic degradation of intravascular clots can be safely obtained with plasmin, it may prove to be a valuable therapeutic agent in certain cases of thromboembolism.

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ANTIHEMOPHILIC FACTOR DEFICIENCY IN THE FEMALE

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THE present report concerns the identification of antihemophilic factor (AHF) deficiency in a female child suffering from a hemorrhagic disorder clinically resembling classic hemophilia. This case is of interest because of its rarity, and serves to illustrate the use of some of the newer laboratory procedures in the diagnosis of obscure coagulation problems.

Case Report.—D. S., M.G.H. 775856, propositus. This 6-year-old white girl was admitted to the hospital for the removal of her tonsils and adenoids on June 16, 1955. There was a past history of easy bruising and of excessive bleeding following dental extractions. The family history revealed that the maternal grandfather was said to be a "bleeder." Physical examination revealed a 20 kilogram girl, who was normal in appearance except for markedly enlarged tonsils and a small hematoma in the right cheek. The concentration of hemoglobin was 12 Gm. per 100 ml., there were 7,500 white cells per cubic millimeter, and the differential cell distribution was normal. The bleeding time was 4½ minutes by the Jacobson method,[3] and the clotting time was 17 minutes in the third tube by the method of Lee and White.[1]

In view of the normal laboratory findings a tonsillectomy and adenoidectomy were decided upon, but the patient was first treated daily with Adrenosem,† 5 mg. given orally, for one week prior to surgery because the past history and the family history were suggestive of a bleeding diathesis. Immediately following the surgery there developed profuse hemorrhage which could not be controlled by the application of an adenoid pack. The administration of 1,000 ml. of fresh whole blood was required to control the bleeding and restore the blood lost during the first 48 hours postoperatively. The patient was discharged on the third postoperative day. Because constant, uncontrollable oozing from the operative site occurred at home the patient was readmitted on the fifth postoperative day. One liter of fresh whole blood was administered during the next 48 hours and the hemorrhage ceased.

History of the maternal grandfather of the propositus: C. M., aged 67, was diagnosed as a hemophiliac at the age of 10 years. Early in life it was noticed that extensive bleeding followed minimal trauma to the nose and other areas of the body. Cuts and minor wounds resulted in profuse hemorrhage which would last for many weeks and could be controlled only by the administration of fresh blood. Minor surgery was accompanied by profuse, almost catastrophic, hemorrhage. Hematomas were frequent, as were hemarthroses. The patient, however, was able to lead a productive life and was

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†An adrenochrome derivative manufactured by the Massengill Corporation.

not crippled. Troublesome hemorrhages occurred frequently until the age of about 50, when they became much less frequent and less severe. Other treatment, which proved to be of temporary benefit, consisted of injections of rabbit serum, horse serum, and serum from his wife.

MATERIALS AND METHODS

A. *Coagulation Studies.*—Blood was obtained from a vein in the antecubital fossa using ordinary syringes and needles. After venipuncture the first several milliliters of blood obtained were discarded and the remainder collected in a separate syringe. When plasma was required, 9 volumes of blood were mixed with one volume of 0.1 M sodium oxalate, and the cells sedimented* at 2,000 r.p.m. for 20 minutes in ordinary glass tubes.

Clotting times of whole blood were determined at 37° C. in 12 × 100 mm. serologic test tubes by a modification of the method of Lee and White.[1]

Platelets were counted by the phase microscopy method of Brecher and Cronkite.[2]

Bleeding times were determined by Ivy's technique as modified by Jacobson.[3]

Prothrombin time was determined by the method of Quick.[4] Specific prothrombin determination in the plasma and in the serum was carried out by Alexander's modification of the Owren method.[5]

Thromboplastin generation tests were done by the procedure of Biggs and Douglas[6] with the following modifications: (1) Plasma was treated with barium sulfate rather than aluminum hydroxide and (2) lyophilized platelet material† was used instead of the subject's platelets, after it had been demonstrated that the subject's platelets were normal. (3) 0.3 ml. aliquots were used, rather than 0.6 ml. to conserve the limited supply of the patient's plasma.

Plasma antihemophilic factor activity was assayed by means of the thromboplastin generation test. A group of 6 curves was constructed, each representing the results of the thromboplastin generation test carried out over a 10-minute period. Six different incubation mixtures were used, representing 100, 50, 16, 8, 1, and 0 per cent AHF activity, respectively. These were obtained as follows: fresh plasma was obtained from 10 normal donors, pooled, absorbed with barium sulfate, and diluted 1:5 with veronal buffer. This pool of plasma was selected to compensate for the wide variation in antihemophilic factor activity in normal individuals. This pooled plasma was designated as containing 100 per cent AHF. Plasma from 3 clinically severe hemophiliacs was pooled and treated similarly. This pooled hemophilic plasma was considered to contain 0 per cent AHF. Four mixtures of these plasmas were made in such proportions that each contained concentrations of 50, 16, 8, and 1 per cent AHF activity, respectively. Separate thromboplastin generation tests were then carried out, using lyophilized platelet material, normal serum, and each of the four plasma mixtures. In each test the observed clotting time of the incubation mixture was plotted against time of incubation. Each of the 6 curves represented a known amount of AHF activity, and this graph served as a basis for estimating the AHF activity in unknown samples (Fig. 1). This method of depicting the curve of thromboplastin generation was preferred to the usual method of expressing per cent thromboplastin formed per minute of incubation because it expresses results which may readily be compared with the data of other workers.

The principle of the thromboplastin generation test depends on the fact that the presence of thromboplastin in maximal amounts will lead to fibrin formation in the substrate in 8 to 12 seconds, after 3 to 5 minutes of incubation. When a reduced rate of thromboplastin generation is present a longer period of incubation is required to obtain clotting times of 8 to 12 seconds. If an unknown sample of blood presents an abnormal thromboplastin generation, then a diagnostic substitution process is carried out wherein plasma, serum, or platelets known to be normal are substituted for each of

*International centrifuge, Model BP, equipped with a four-place head and 550 ml. cups; available from International Equipment Company, Boston, Mass.

†Kindly supplied by Dr. Edmund Klein, Children's Cancer Research Foundation, Children's Medical Center, Boston, Mass.

TABLE I. THE COAGULATION PROFILE OF THE PATIENT AND FAMILY MEMBERS COMPARED TO THAT OF A KNOWN SEVERE HEMOPHILIAC

	NORMAL RANGE	PATIENT D. S.	FEMALE SIBLING N. S.	MOTHER MRS. S.	FATHER MR. S.	MRS. M. MATERNAL GRANDMOTHER	MR. M. MATERNAL GRANDFATHER	W. A. SEVERE TYPICAL HEMOPHILIAC
1. Platelet Count	250-300,000 c.mm.	310,000	360,000	348,000	298,000	307,000	280,000	310,000
2. Clotting Time	Up to 20 min. in third glass tube	8, 12, 18 min.	8, 9, 10 min.	9, 10, 11 min.	7, 10, 13 min.	10, 12, 14 min.	19, 23, 29 min.	60, 90, 120 min.
3. Bleeding Time	Up to 6½ min.	4½ min.	5 min.	3 min.	4½ min.	5 min.	4 min.	5 min.
4. Prothrombin Time (Quick)	Up to 13 sec.	12½ sec.	13 sec.	13 sec.	12½ sec.	12 sec.	13 sec.	12½ sec.
5. Prothrombin Time (Owren)	Up to 15.5 sec.	15.0 sec.	15.2 sec.	15.5 sec.	14.9 sec.	15.0 sec.	15.5 sec.	15.3 sec.
6. AC Globulin	Up to 19.2 sec.	19 sec.	19.5 sec.	18.9	19.2	19.9	19.0	18.8
7. SPCA	30.3 sec.	30.3	29.8	31.0	30.0	30.8	30.5	31.0
8. Prothrombin Consumption	Between 25-31 sec.	28	31	30	29.5	29	33	14.5
9. Fibrinogen	250-400 mg.	320	---	---	---	---	300	360
10. Circulating Anticoagulant	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
11. Thromboplastin Generation	Clot in 2-4 min.	11 min.	3 min.	4 min.	3 min.	3 min.	15 min.	18-20 min.
12. Plasma AHF	70-125%	6%	90%	100%	100%	95%	2%	0%

This table shows the results of the coagulation studies performed on the patient and her family. Notice the abnormality in the thromboplastin generation test and the plasma AHF assay of the patient and maternal grandfather. Data from a severe male hemophiliac are included for comparison.

these respective blood components from the patient. After the site of the defect has been localized, i.e., in the serum, plasma, or platelets, specific identification procedures are followed. In these, the corrective effects of the unknown blood are observed on the abnormal thromboplastin generation of blood from a patient whose specific deficiency* (PTA, PTC, AHF) is known. If the unknown blood can correct the thromboplastin generation defect of the known defective blood, it is concluded that their defects differ. By this process it is possible to identify a specific defect.

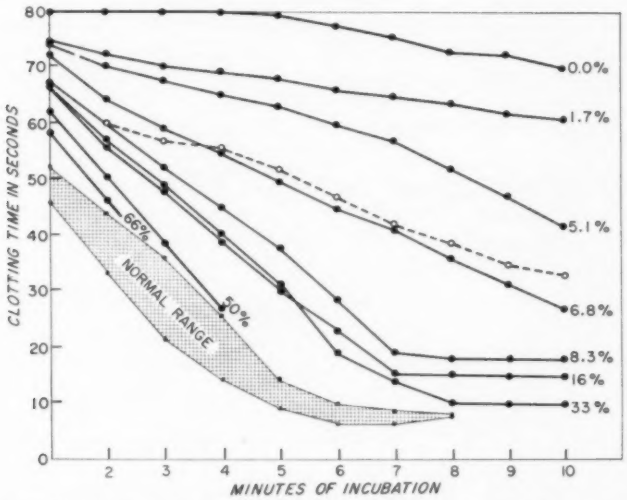

Fig. 1.—Thromboplastin generation tests showing comparison between curve of Patient D. S. (– – –) and curves made from samples of blood containing known amounts of AHF (————). The figure demonstrates the degree of AHF deficiency (6 per cent) in the patient, using the thromboplastin generation method of assay.

Capillary fragility was tested by maintaining a blood pressure cuff on the arm at a pressure midway between the systolic and diastolic pressures for 5 minutes.

AC Globulin was determined by the method of Alexander.[7] SPCA was determined by the method of Alexander.[8]

The presence of circulating anticoagulants was investigated by the method of Stefanini.[9]

B. *Special Studies.*—The circulating polymorphonuclear leukocytes and the epithelial cells of the buccal mucous membranes were examined to determine the patient's genetic sex.[10, 11]

The blood types and the electrophoretic patterns of plasma of the family members[17] were determined.

RESULTS

A. *Laboratory Findings.*—The results of the laboratory findings on the patient, her maternal grandfather, and the available family members are shown in Table I. Tests 1 through 10 of the table demonstrate that with the exception of the slightly prolonged clotting time of the maternal grandfather, the clotting pattern of the patient and of her family is normal. The thromboplastin generation test and the plasma AHF assay (tests 11 and 12, Table I), however, clearly

*PTA = Plasma thromboplastin antecedent; PTC = Plasma thromboplastin component; AHF = Antihemophilic factor deficiency.

delineated the quality and quantity of the coagulation defect present in the patient and her grandfather. In the thromboplastin generation test the patient's plasma appeared to be similar to that of a male with mild, classical hemophilia who was being studied at the same time (Figs. 1 and 2). The studies of the patient's ability to generate thromboplastin under a wide variety of conditions demonstrated that the only detectable abnormality is a deficiency of AHF (Table II) and that she does not have a deficiency of either plasma thromboplastin antecedent (PTA) or component (PTC). The plasma AHF assay described above indicated that the patient had about 6 per cent AHF (Table I) and her grandfather had about 2 per cent.

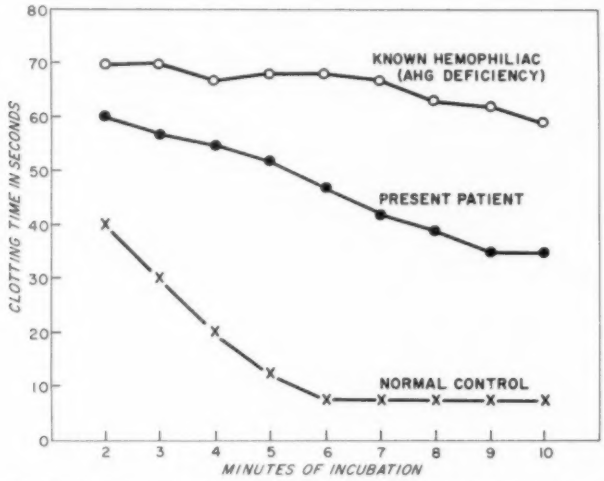

Fig. 2.—Thromboplastin generation tests showing comparison between normal control, known hemophiliac, and present patient.

Samples of blood from the patient and members of her family, unidentified as to name and sex, were sent to other laboratories for confirmative testing. Dr. Robert Goldstein of the Beth Israel Hospital, Boston, found that the patient's and her grandfather's plasma were not able to correct completely the prothrombin consumption defect of a known severe classical hemophiliac (Table III), while the plasma of the other members of the family did so. Dr. Stanley Porter of the Children's Medical Center, Boston, studied the patient and her grandfather and found that each had an abnormal thromboplastin generation test which was correctable by normal plasma but which was not correctable by the addition of the other's plasma.

Thirty per cent of a sample of cells from the patient's buccal mucosa showed the chromatin masses characteristic of female cells.[10] Twenty per cent of the polymorphonuclear leukocytes in a smear of the patient's peripheral blood showed the drumstick structure, characteristic of the cells of females.[11] Hence, the cytologic studies confirm the conclusion reached on morphologic grounds that the patient is female.

Bernfeld[17] has discovered an abnormality in the electrophoretic pattern of the plasma of patients with a variety of hemorrhagic disturbances as well as family members of some classical hemophiliacs, which he has termed the alpha-X anomaly. The abnormality, which is much more pronounced in the ascending boundaries than in the descending boundaries, consists of: (1) a sharply pointed peak (alpha-X) which migrates faster than the usual alpha-2 globulin. (2) The disappearance of the usual alpha-2 and alpha-3 globulin peaks. (3) A pronounced asymmetry to the shape of the alpha-X peak. Electrophoretic analysis of the plasmas of the family members was done to test for the alpha-X anomaly.* The anomaly was not found in the electrophoretic patterns of any of the family members. The patterns of the patient's sibling, N. S.; mother, Mrs. S.; maternal grandmother, Mrs. M.; and maternal grandfather, Mr. M., were normal and similar in appearance. The patient's pattern, while normal, differed markedly from that of the others in that the alpha-1 globulin concentration was moderately elevated, the alpha-2 globulin concentration was greatly elevated, but the concentrations of the beta-1 and gamma globulin were low.

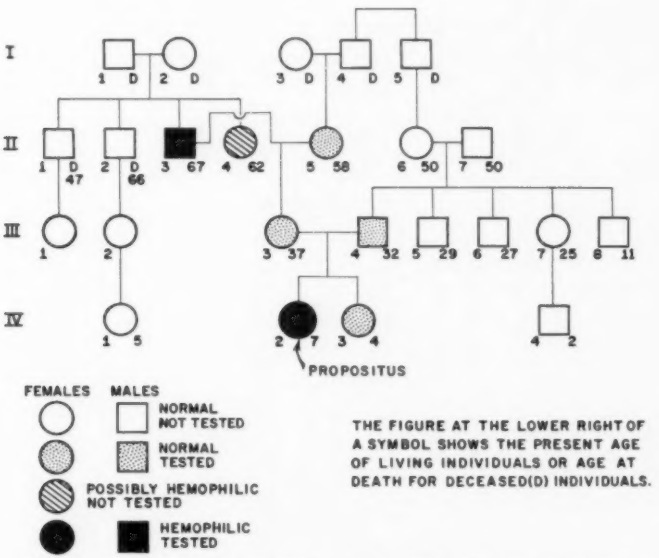

Fig. 3.—Pedigree of the family of a female child with AHF deficiency.

B. *Inheritance.*—Classical hemophilia (AHF deficiency) is inherited via a sex-linked recessive gene; hence an affected female would be expected to have an affected father. The clinical and laboratory data, however, indicate that the patient's father is not affected. Some consideration, therefore, must be given to other possible genetic explanations of the pedigree (Fig. 3).

Information for the pedigree was obtained by direct interviews with II-3, II-5, III-3, and III-4; III-2 was interviewed by telephone. Only II-3, II-5,

*Kindly performed by Dr. Peter Bernfeld of the New England Center Hospital.

TABLE II. EFFECT ON THROMBOPLASTIN GENERATION OF MIXTURES OF THE PATIENT'S PLASMA OR SERUM WITH THE PLASMA OR SERUM OF INDIVIDUALS KNOWN TO BE DEFICIENT IN A SPECIFIC COAGULATION FACTOR

INCUBATION MIXTURE		MINUTES OF INCUBATION							INTERPRETATION
		2	3	4	5	6	7	8	
1. Control plasma 0.3 c.c.	Clotting time in seconds	25	14	10	9	10	10	11	Normal thromboplastin generation
2. Patient's plasma 0.3 c.c. Patient's serum 0.3 c.c.	Clotting time in seconds	60	57	55	52	47	42	39	Defective thromboplastin generation
3. Control Plasma 0.3 c.c. Patient's serum 0.3 c.c.	Clotting time in seconds	24	14	12	9	9	9	10	The addition of control plasma corrects patient's defective thromboplastin generation
4. Patient's plasma 0.3 c.c. Control serum 0.3 c.c.	Clotting time in seconds	62	58	57	46	42	38	36	The addition of control serum has no effect on the defective thromboplastin generation of the patient
5. AHF deficient plasma 0.3 c.c. Control serum 0.3 c.c.	Clotting time in seconds	70	70	67	68	68	67	63	Defective thromboplastin generation (AHF deficiency)
6. Patient's plasma 0.15 c.c. AHF deficient plasma 0.15 c.c. Control serum 0.3 c.c.	Clotting time in seconds	76	78	76	68	64	62	60	The mixture of the patient's plasma and known AHF deficient plasma, in equal amounts, does not correct the defective generation of the hemophiliac, indicating that the patient lacks AHF in her plasma
7. Control plasma 0.3 c.c. PTC deficient serum 0.3 c.c.	Clotting time in seconds	38	34	30	32	30	26	22	Defective thromboplastin generation (PTC deficiency)

TABLE II—CONT'D

8. Control plasma 0.3 c.c. Patient's serum 0.15 c.c. PTC deficient serum 0.15 c.c.	Clotting time in seconds	32	16	14	12	10	9	8	The mixture of the patient's serum and known PTC deficient serum, in equal amounts, does correct the defective generation of the known PTC—thus the patient has normal amounts of PTC in her serum
9. PTA deficient plasma 0.3 c.c. PTA deficient serum 0.3 c.c.	Clotting time in seconds	34	28	26	20	20	20	20	Defective thromboplastin generation (PTA deficiency)
10. Patient's plasma 0.15 c.c. PTA deficient plasma 0.15 c.c. PTA deficient serum 0.3 c.c.	Clotting time in seconds	28	16	13	10	9	9	9	The mixture of the patient's plasma and known PTA deficient plasma, in equal amounts, does correct the defective generation of the known PTA—thus the patient has normal amounts of PTA in her plasma
11. PTA deficient plasma 0.3 c.c. PTA deficient serum 0.15 c.c. Patient's serum 0.15 c.c.	Clotting time in seconds	30	15	12	9	9	8	9	The mixture of the patient's serum and known PTA deficient serum, in equal amounts, does correct the defective generation of the known PTA—thus the patient has normal amounts of PTA in her serum

This table shows the results of the thromboplastin generation tests by means of which the specific deficiency (AHF) in the patient was delineated.

III-3, III-4, IV-2, and IV-3 were examined. II-4 was reported by II-3 and II-5 to be a bleeder, but her hospital record showed no evidence to support this and she denied, over the telephone, that she was a bleeder; but this woman, who is a recluse, refused to be examined or to permit us to visit her. III-2 denied any knowledge of a bleeding tendency in herself or any of her relatives. She was certain that neither her father nor his mother was a bleeder. This assertion is in agreement with the statements of II-3 and II-5.

TABLE III. EFFECT OF 0.1 ML. OF PLASMA FROM VARIOUS DONORS ON THE PROTHROMBIN CONSUMPTION OF 2 ML. OF AHF DEFICIENT WHOLE BLOOD

DONOR	RESIDUAL SERUM PROTHROMBIN CONTENT OF AHF DEFICIENT WHOLE BLOOD (PER CENT)
Patient, D. S.	26.5
Sibling, M. S.	2.2
Mother, Mrs. S.	8.0
Maternal grandmother, Mrs. M.	4.0
Maternal grandfather, Mr. M.	23
Saline	88

Note the partially corrective effect of the patient's and grandfather's plasma on the prothrombin consumption of a severe hemophiliac (AHF). This would suggest that their deficiencies are not extreme.

The fact that the patient's parents are second cousins (their mothers being first cousins, and their maternal grandfathers being siblings) seems to be of no significance, because the AHF deficiency is introduced via the family of the maternal grandfather and this family is unrelated to the other.

The hemophilia suffered by the patient's grandfather (II-3) is mild, similar to that described by Graham and associates[15] in which some heterozygotes show reduced AHF. Hence, a possible explanation of the pedigree is that both the patient and her mother are heterozygous for the gene which gave rise to the grandfather's hemophilia, and that they represent the opposite extremes of variation of AHF content in heterozygotes. This would be analogous to the situation in cystic fibrosis of the pancreas in which the concentration of electrolytes in the sweat of the heterozygotes varies from the low normal range well into that of the patients. The alleged bleeding tendency of II-4 would be in agreement with this assumption.

Another possibility is that the patient is the child of a mating between her mother and her maternal grandfather. This possibility was eliminated by comparing the blood types of the members of the family (Table IV). The patient is R_1r while her mother is rr, her grandfather is R_2r, and her legal father R_1R_2. Clearly the child could not have resulted from a mating of her mother and grandfather, but she could have been the offspring of her legal parents.

The pedigree does not exclude the possibility that the hemophilia in this family is due to an autosomal dominant with incomplete penetrance nor, for that matter, does it exclude autosomal recessive inheritance. The latter possibility, however, would require that I-1 and I-2 both be heterozygous for the gene involved and that III-4 also be heterozygous for this same gene. While it is possible for 3 unrelated people in a single pedigree to be heterozygous for

what would have to be a very rare gene, it is highly improbable that such is the case. Similarly it is highly improbable that a sperm, bearing a mutation to the recessive allele for hemophilia, fertilized an egg carrying this allele. Finally, various bizarre chromosomal anomalies known to arise in animals other than man could be postulated. The most likely explanation, however, seems to be that the patient is heterozygous for an allele of classical hemophilia and exhibits an extreme AHF deficiency. The explanation cannot be tested until the patient has offspring and then only if she has a normal son.

TABLE IV. BLOOD TYPES OF THE PATIENT AND HER FAMILY

	A	B	C	D (Rh)	E	c	e	C^w	M	N	S	K	Fy^a	Jk^a	P	Le^a	Le^b
Female patient, D. S.	–	–	+	+	–	+		–	+	–	+	–	–	–	+	–	+
Female sibling, N. S.	–	–	–	+	+	+	+	–	+	–	+	–	–	+	+	–	+
Mother, Mrs. S.	–	–	–	–	–	+		–	+	+	+	–	+	–	+	–	+
Father, Mr. S.	–	–	+	+	+	+	+	–	+	–	+	–	+	+	+	–	+
Mrs. M., maternal grandmother	–	+	–	–	–	+		–	–	+	–	–	+		+	–	+
Mr. M., maternal grandfather	+	–	–	+	+	+	+	–	+	–	+	–	–		+	–	–

+ = Positive; – = negative.

Inspection of this table indicates that the patient, D. S., has one Rh subgroup, C, which is common to none of the others tested except her father, Mr. S.

This table records the results of the blood grouping studies on the family which indicate that the maternal grandfather could not be the biologic father of the patient.

DISCUSSION

A consideration of reported hemorrhagic conditions from which females suffer did not provide an explanation of the diagnosis in this case. It was evident that this patient did not belong in that group of cases first described by Alexander and Goldstein[13] and later by Shulman and Smith and others,[14, 19] which exhibits a dual hemostatic defect, namely, a prolonged bleeding time in combination with reduced amounts of plasma AHF. Furthermore, it is unlikely that she had an acquired defect such as described by O'Brien and associates.[16] The medical literature relating to the detection of hemophilic carriers by laboratory techniques is conflicting. The basis for the conflict may reside in the differences of techniques employed and in the failure in the past to differentiate the hemophilioid diseases from classical hemophilia. This subject has been recently summarized by Margolius and Ratnoff.[12] Nonetheless, it is clear that in a few instances one may find female carriers who have minor bleeding manifestations without demonstrable laboratory abnormalities of coagulation, or, on the other hand, one may find asymptomatic carriers with slight aberrations in their clotting mechanism which can be detected only by sensitive

laboratory techniques.[12] The present patient appears to have a combination of these previously reported findings, i.e., definite hemorrhagic manifestations and abnormalities in coagulation which are easily detectable in the laboratory. In short, this female child appears to have clinical hemophilia as well as laboratory hemophilia, i.e., AHF deficiency.

The possible genetic explanations for the occurrence of hemophilia in this patient have been discussed above. It seems most likely that the patient is an example of a carrier of an allele leading to a mild form of hemophilia with a reduced amount of plasma AHF, such as described by Graham and co-workers.[15, 18] She is not identical with these cases, however, in that she has clinical hemorrhagic tendencies which were not present in the female carriers detected by Graham and associates. Nonetheless, a number of similarities and some differences between this family and that of Graham's should be noted. The maternal grandfather in the family under discussion has classical hemophilia of a mild type. The diagnosis of hemophilia is based on the fact that he is a male, that his clotting time is prolonged, and that he has a deficiency of plasma AHF. His advanced age and lack of crippling attest to the clinical mildness of his condition. While hemarthroses have occurred, he has survived, and he has withstood hemorrhoidectomy and herniorraphy successfully albeit with the assistance of numerous blood transfusions. His clinical status is somewhat similar to that of the male members of the family described by Graham and associates who have a mild form of hemophilia. In contrast to the findings in the members of the latter family, in whom the laboratory defect was so slight that without the very sensitive AHF assay it would have been difficult to detect it, the defect in our patient is marked and easily detected. D. S., the subject of this report, demonstrated very much the same clinical course as her grandfather. From the laboratory viewpoint, her clotting defect was less extensive than his (her clotting time was normal) and her plasma AHF deficiency was not as marked. It should be noted, however, that attention was first called to her because of extensive hemorrhage following minor surgery. It must be remembered that the AHF assay used in this study is not a quantitative determination in the chemical sense of the term, and for this reason it is hard to be exact concerning the concentration of AHF in these patients.

Thus, the results of clinical, laboratory, and genetic studies most closely coincide with those of a female carrier of classical hemophilia, who has a detectable coagulation defect. The data which have been presented would support the suggestion that the mutant gene carried by the present patient is incompletely recessive, and that she has a degree of clinical expression of hemophilia not observed or previously reported in female carriers.

It is clear from the foregoing that there are a number of clinical situations which can result in a deficiency of plasma AHF in females. It would perhaps be wise to restrict the term "hemophilia in the female" to those individuals who can be shown to be homozygous for the sex-linked gene leading to classical hemophilia in the male. Others, such as the one described in this paper, might best be referred to as "detectable carriers."

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CONGENITAL COAGULATION DEFICIENCY OF STUART FACTOR ACTIVITY

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THE complex nature of the prothrombin activity in the coagulation scheme has been intensively studied in the past decade. For optimal conversion of prothrombin to thrombin, 3 factors have been generally accepted as necessary: prothrombin, a labile factor (factor V, proaccelerin, or accelerator globulin), and a stable factor (factor VII, proconvertin, or serum prothrombin conversion accelerator).[1-5] Recently, Hougie, Barrow, and Graham[6] have identified an additional activity, tentatively called "Stuart" factor. This activity is present in normal plasma and serum but not in plasma adsorbed with barium sulfate. A deficiency of Stuart factor has been defined in a patient who was previously described as having a congenital hypoproconvertinemia (Lewis and associates).[7] Hougie's differentiation between proconvertin (SPCA) and Stuart factor was based upon the observation that his patient had an abnormal thromboplastin generation test and a prolonged Russell viper venom (Stypven) time, whereas these tests were normal in reported cases of proconvertin deficiency. Deficiencies of both factors had in common a prolonged Quick prothrombin time and a delayed prothrombin conversion (two-stage prothrombin time) which were corrected by normal serum but not by barium sulfate adsorbed plasma. Experiments in which plasmas from Stuart factor and proconvertin deficiencies were mixed demonstrated that these two plasmas were mutually corrective in the prothrombin time test.

The present study is an investigation of a patient (J. L.) who was first reported by Hagen and Watson[8] as idiopathic familial hypoprothrombinemia. Later, Frick and Hagen[9] defined this patient's deficiency as one of stable prothrombin conversion factor. We now believe that the evidence indicates the deficiency to be one of Stuart factor activity.

In a re-evaluation of this patient, an abnormality of the thromboplastin generation test and a prolonged Russell viper venom time were observed. When plasma from Owren's patient with proconvertin deficiency was mixed with plasma from our patient, a correction of their respective prolonged prothrombin times was observed. When serum from Hougie's patient with Stuart factor deficiency was mixed with plasma from our patient, no correction occurred.

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The purpose of this study is to present evidence that the deficiency present in our patient is not that of proconvertin but is consistent with a deficiency of Stuart factor.

MATERIALS

Plasma Samples.—Venous blood from the patient and from normal controls was mixed 9 parts to 1 with sodium oxalate 0.1 M or with sodium citrate 3.13 per cent and centrifuged at 3 000 r.p.m. for 15 minutes.

Proconvertin deficient plasma from Owren's patient was obtained in the lyophilized state through the courtesy of Dr. Peter F. Hjort.

Stuart factor deficient serum was obtained in the lyophilized state through the courtesy of Dr. John B. Graham.

Plasma free from prothrombin, proconvertin, and Stuart factor was obtained by mixing human or beef oxalated plasma with barium sulfate, 100 mg. per ml. plasma, for 15 minutes. The adsorbed plasma was separated after centrifuging at 3,000 r.p.m. for 15 minutes.

Serum containing stable activities was obtained from normal blood to which thromboplastin had been added. The serum, after separation from the clot, was stored for 48 hours. For use in the Russell viper venom-cephalin prothrombin assay, serum similarly obtained was stored for 4 days, mixed with 1:10 volume sodium oxalate 0.1 M, and adsorbed with $BaSO_4$, 100 mg. per milliliter of serum. The adsorbate was eluted with sodium citrate, the eluate was dialyzed for 20 hours against sodium chloride and the pH was adjusted to 7.4.[10] The solution was stored at -20° C.

Platelets were prepared from blood mixed with sodium EDTA as anticoagulant. The blood was centrifuged slowly, the platelets were removed and washed 3 times with saline, and the suspension was reconstituted to one-third the original plasma volume. Siliconized syringes, needles, and glassware were used in all manipulations with platelets.

*Rabbit brain thromboplastin** was used throughout.

Russell viper venom (Stypven)† was used in a concentration of 1:10,000 (10 mg. per 100 ml.) in the Russell viper venom times, with or without thromboplastin. In the specific assay for prothrombin, using Russell viper venom in combination with cephalin, the concentration of viper venom was 1:40,000.

Cephalin was prepared according to the method of Hjort, Rapaport, and Owren.[11] The crude cephalin was extracted with ether from acetone-dried human brain, suspended in veronal buffer and stored at -20° C. In the partial thromboplastin test, cephalin was used in a concentration of 0.3 per cent. In the specific assay for prothrombin in combination with Russell viper venom, cephalin was used in a concentration of 0.15 per cent.

METHODS

Whole blood clotting time was done by a modification of the method of Lee and White, using siliconized syringes and needles but nonsiliconized glass test tubes.

One-stage prothrombin times were done according to the method of Quick.[12]

Two-stage prothrombin determinations (prothrombin conversion rates) were done as described by Frick.[9] The test plasma was diluted 0.1 ml. in 2.6 ml. saline; 0.9 ml. of this plasma dilution was pipetted into the reaction mixture which consisted of 1.6 ml. saline, 0.8 ml. acacia, 0.15 ml. imidazole buffer, and 0.15 ml. thromboplastin. At intervals of 1 to 2 minutes, 0.4 ml. of the reaction mixture was pipetted into a test tube and mixed with 0.1 ml. fibrinogen 0.5 per cent.

*Difco Laboratories, Detroit, Mich.
†Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.

Prothrombin consumption was estimated with the two-stage prothrombin technique, by measuring the residual prothrombin activity 30 and 60 minutes after phlebotomy.[9] Prothrombin consumption was also estimated with Russell viper venom-cephalin prothrombin method by measuring the serum prothrombin 60 minutes after phlebotomy.

Russell viper venom times and Russell viper venom-thromboplastin times were done as described by Hougie, Barrow, and Graham.[6]

Thromboplastin generation tests were done as described by Biggs and Douglas.[13]

Partial thromboplastin times were done according to the method of Langdell, Wagner, and Brinkhous.[14] One-tenth milliliter oxalated plasma was mixed with 0.1 ml. cephalin 0.3 per cent, and the clotting time at 37° C. was measured after the addition of 0.1 ml. $CaCl_2$ 0.01 M.

Proconvertin assay was done using proconvertin deficient plasma as a substrate. One-tenth milliliter thromboplastin, 0.1 ml. adsorbed beef plasma, 0.1 ml. proconvertin deficient plasma, and 0.1 ml. test plasma diluted 1:10 were mixed; the clotting time at 37° C. was measured after the addition of $CaCl_2$ 0.02 M.

Russell viper venom-cephalin method for prothrombin assay was done as described by Hjort, Rapaport, and Owren.[11] One-tenth milliliter Russell viper venom 1:40,000 in cephalin 0.15 per cent was mixed with 0.1 ml. adsorbed beef plasma and 0.1 ml. test plasma diluted 1:50, and the clotting time at 37° C. was measured after the addition of $CaCl_2$ 0.035 M.

A modification of this method[15] was also used, which introduced into the test system an eluate from the barium sulfate adsorbate from normal serum. The test system was the same except that $CaCl_2$ was mixed 4 parts to 1 with the serum eluate, and this mixture was added in place of the $CaCl_2$ alone.

Per cent prothrombin activity was read from a correlation graph prepared for the respective test systems.

TABLE I. ONE-STAGE PROTHROMBIN (QUICK) TIMES

NORMAL PLASMA	SALINE	NORMAL ADSORBED PLASMA	NORMAL SERUM ELUATE	J. L. PLASMA	PROCONVERTIN DEF. PLASMA	STUART FACTOR DEF. SERUM	CLOTTING TIME (SEC.)
0.05	0.05						21.0
	0.05			0.05			54.0
0.05				0.05			17.8
		0.05		0.05			49.5
			0.05	0.05			13.0
	0.05				0.05		80.0
0.05					0.05		20.6
				0.05	0.05		18.0
	0.05					0.05	α
0.05						0.05	23.0
				0.05		0.05	55.0
					0.05	0.05	25.0

Test system: 0.1 ml. thromboplastin; 0.1 ml. plasma as indicated; 0.1 ml. $CaCl_2$ 0.015 M.

RESULTS

The whole blood clotting time at the time of these investigations was 23 minutes; the normal range in our laboratories is 10 to 20 minutes.

One-Stage Prothrombin Times.—The Quick time was prolonged, and was corrected by normal plasma and by normal aged serum but was not corrected by fresh barium sulfate adsorbed plasma (Table I). When the patient's plasma was mixed with plasma from a patient with proconvertin deficiency, a normal Quick time resulted although both these plasmas by themselves had markedly prolonged Quick times. When serum from a patient with a deficiency of Stuart

factor was mixed with proconvertin deficient plasma, a normal Quick time again resulted which demonstrated a mutual correction between these deficiencies. According to Hougie and associates[6] the Quick time of Stuart plasma was prolonged. When the patient's plasma was mixed with Stuart deficiency serum, the Quick time remained prolonged, which indicated that the patient's plasma and Stuart deficiency serum had a common deficiency.

Two-Stage Prothrombin Determinations.—The two-stage prothrombin conversion rate was delayed although the minimal clotting time did eventually become normal (Fig. 1). This indicated that although the rate of prothrombin conversion was delayed, the total amount of thrombin formed was normal and thus the amount of prothrombin was normal. The rate of prothrombin conversion, as measured by the two-stage prothrombin method, was corrected by normal aged serum but not by fresh barium sulfate adsorbed plasma (Fig. 1).

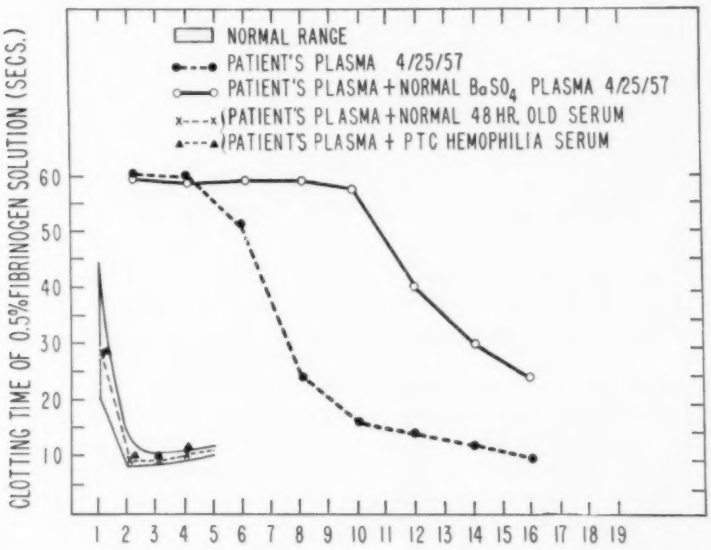
NORMAL RANGE
PATIENT'S PLASMA 4/25/57
PATIENT'S PLASMA + NORMAL $BaSO_4$ PLASMA 4/25/57
PATIENT'S PLASMA + NORMAL 48 HR. OLD SERUM
PATIENT'S PLASMA + PTC HEMOPHILIA SERUM
CLOTTING TIME OF 0.5% FIBRINOGEN SOLUTION (SECS.)
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Fig. 1.—Prothrombin conversion (two-stage technique) in minutes.

The abnormal rate of prothrombin conversion of our patient's plasma was corrected by serum from a patient with a deficiency of plasma thromboplastin component (PTC or hemophilia B) (Fig. 1).

The two-stage prothrombin determinations showed some variation on 3 separate occasions. However, at no time was the prothrombin conversion within the normal limits. With this method, the normal clotting time of 10 seconds is usually reached between 2 and 4 minutes. In our patient, a clotting time of 10 to 12 seconds was not reached until 6 to 18 minutes.

The plasma from the proconvertin deficient patient demonstrated a markedly delayed prothrombin conversion rate (Fig. 2). These abnormalities

in the rate of prothrombin conversion in plasmas from our patient and from the patient with a deficiency of proconvertin were corrected to the normal range of prothrombin conversion when their respective plasmas were mixed. However, serum from the patient with Stuart factor deficiency was not able to correct the delayed prothrombin conversion of our patient's plasma.

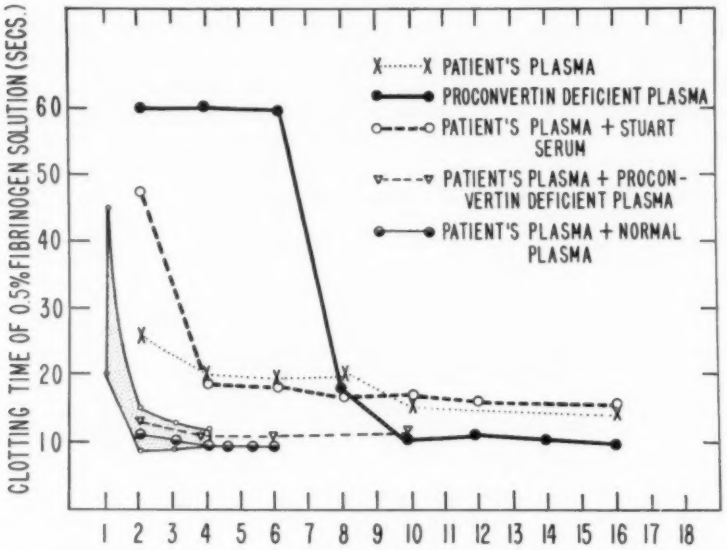
CLOTTING TIME OF 0.5% FIBRINOGEN SOLUTION (SECS.)
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
X·····X PATIENT'S PLASMA
PROCONVERTIN DEFICIENT PLASMA
PATIENT'S PLASMA + STUART SERUM
PATIENT'S PLASMA + PROCONVERTIN DEFICIENT PLASMA
PATIENT'S PLASMA + NORMAL PLASMA

Fig. 2.—Prothrombin conversion in minutes.

Prothrombin Consumption.—Frick and Hagen[9] noted previously that this patient had an adequate and normal consumption of prothrombin by the two-stage method. This was confirmed in the present studies. When prothrombin consumption was measured in the RVV-cephalin test system, 76 per cent of the plasma prothrombin was consumed (Table II). This would appear to be at the lower limits of the normal range.

Table II. Prothrombin Consumption

METHOD	PLASMA PROTHROMBIN (PER CENT)	SERUM PROTHROMBIN (PER CENT)	PER CENT PLASMA PROTHROMBIN CONSUMED
RVV-cephalin prothrombin	90	20	70/90 = 76
Two-stage prothrombin	100	5	95/100 = 95

Thromboplastin Generation Test.—Thromboplastin generation test was abnormal on two occasions (Fig. 3). This abnormality was not corrected when normal barium sulfate adsorbed plasma was substituted for the patient's adsorbed plasma in the incubation mixture. When normal serum was substituted for the patient's serum in this mixture, thromboplastin generation was normal. Normal platelets when used with patient's plasma and serum resulted in an abnormal thromboplastin generation (Fig. 3).

Thromboplastin generation was normal when serum from hemophilia B (PTC) was used 1:1 with patient's serum in the test system (Fig. 4). Substitution of the patient's adsorbed plasma, in the thromboplastin generation test of a hemophilia A (AHG) patient, resulted in a normal test (Fig. 4).

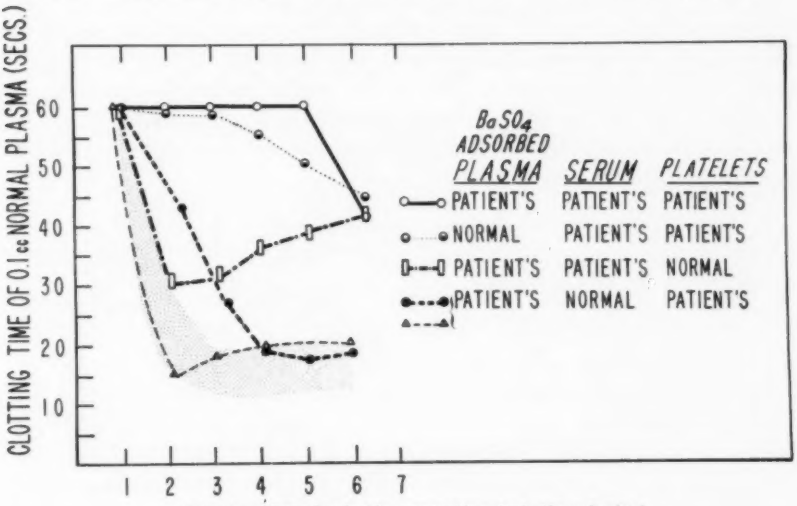

Fig. 3.—Thromboplastin generation test (in minutes).

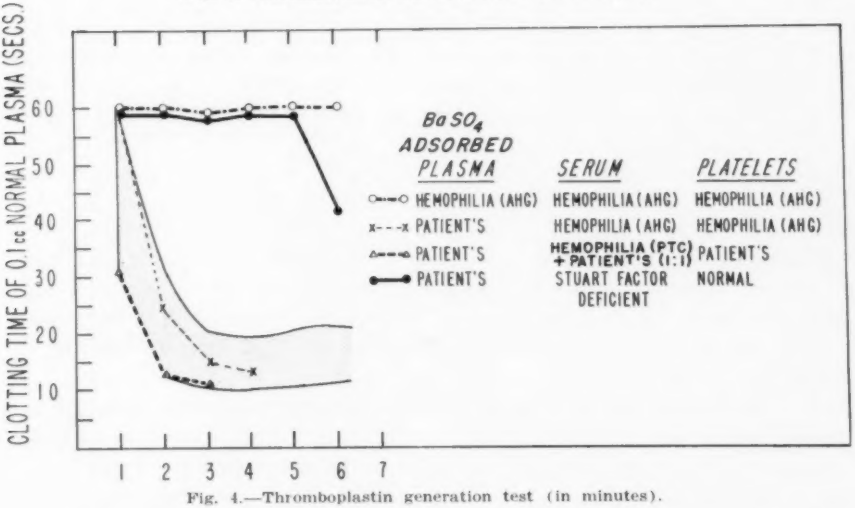

Fig. 4.—Thromboplastin generation test (in minutes).

When Stuart factor deficient serum was substituted for the patient's serum, the thromboplastin generation test remained abnormal (Fig. 4).

Partial Thromboplastin Times.—The normal values in our laboratory ranged from 27.5 to 42.6 seconds. The patient's partial thromboplastin time with cephalin was 94 seconds (Table III).

TABLE III. PARTIAL THROMBOPLASTIN (CEPHALIN) TIMES

Normal range	27.5-42.6 sec.
J. L.	94 sec.

Test system: 0.1 ml. cephalin 0.3 per cent; 0.1 ml. plasma; 0.1 ml. $CaCl_2$ 0.01 M.

Russell Viper Venom.—When only Russell viper venom was used as a partial thromboplastin, the patient's plasma (69.1 sec.) appeared to have significantly less activity than normal plasma (27.6 to 37.0 sec.) (Table IV). The activity of the lyophilized proconvertin deficient plasma (46.4 sec.) was between these extremes. Similar differences were observed when Russell viper venom and saline were used in the test mixtures.

TABLE IV. RUSSELL VIPER VENOM (STYPVEN) TIMES

NORMAL PLASMA	J. L. PLASMA	PROCONVERTIN DEF. PLASMA	STUART F. DEF. SERUM	SALINE	CLOTTING TIME (SEC.)
0.1					27.6-37.0
	0.1				69.1
		0.1			46.4
0.1				0.1	51.5
	0.1			0.1	104.3
		0.1		0.1	62.6
0.05	0.05				43.3
	0.05	0.05			33.6
	0.05		0.05		101.0

Test system: 0.1 ml. Russell viper venom 1:10,000; 0.1 ml. plasma as indicated; 0.1 ml. $CaCl_2$ 0.015 M.

An equal mixture of patient's and normal plasmas gave a result of 43 sec. When the patient's plasma was mixed with an equal volume of proconvertin deficient plasma, the combined activity was slightly greater (33 sec.). A mixture of patient's plasma and Stuart's serum showed a markedly decreased activity (101 sec.) (Table IV).

These tests, although done on lyophilized plasma, demonstrate consistent corrective effects of normal and proconvertin deficient plasma and a lack of correction of Stuart factor deficient serum on our patient's plasma. The slightly abnormal time of the lyophilized proconvertin deficient plasma when used with Russell viper venom alone conceivably might be normal if fresh plasma had been used, but, in present context, this should be compared to the normal times obtained below when lipoid substances are included by addition of cephalin.

Russell Viper Venom and Cephalin or Thromboplastin.—By adding either cephalin or thromboplastin to Russell viper venom, the behavior of the patient's plasma and of proconvertin deficient plasma differed markedly (Table V and VI). The clotting time of proconvertin deficient plasma was the same as that of normal plasma while the clotting time of the patient's plasma was considerably longer.

Russell Viper Venom-Cephalin Modified Method for Prothrombin.—The activities of the respective plasmas were measured in a test that has been thought to

be specific for prothrombin. Hjort[11] has shown that the Russell viper venom-cephalin method was independent of proconvertin. This was confirmed in this study (Table VI). With addition of adsorbed beef plasmas as source of proaccelerin, the test system has been thought to measure only prothrombin activity.[11, 16]

TABLE V. RUSSELL VIPER VENOM-THROMBOPLASTIN TIMES

Normal plasma	7.5 sec.
J. L. plasma	15.2 sec.
Proconvertin deficient plasma	7.5 sec.

Test system: 0.1 ml. Russell viper venom 1:10,000; 0.1 ml. thromboplastin; 0.1 ml. plasma; 0.1 ml. $CaCl_2$ 0.015 M.

TABLE VI. RUSSELL VIPER VENOM AND CEPHALIN PROTHROMBIN ASSAY: ORIGINAL METHOD

Normal plasma	26.3 sec.	95 per cent activity
J. L. plasma	49.2 sec.	33 per cent activity
Proconvertin deficient plasma	23.3 sec.	107 per cent activity

Test system: 0.1 ml. Russell viper venom 1:40,000 in cephalin 0.15 per cent; 0.1 ml. adsorbed beef plasma; 0.1 ml. test plasma diluted 1:50; 0.1 ml. $CaCl_2$ 0.035 M.

However, our patient's plasma, which has been shown above to have a normal amount of prothrombin, had only 33 per cent activity in Hjort's test system (Table VI). A normal prothrombin activity of 90 per cent was obtained only when the test was modified to include the "Stuart factor." This was accomplished by the addition of the eluate of the barium sulfate adsorbable activities present in normal serum to the test system (Table VII).

TABLE VII. RUSSELL VIPER VENOM AND CEPHALIN PROTHROMBIN ASSAY: MODIFIED METHOD

Normal plasma	27.0 sec.	100 per cent activity
J. L. plasma	28.4 sec.	90 per cent activity
Proconvertin deficient plasma	25.2 sec.	106 per cent activity

Test system: 0.1 ml. Russell viper venom 1:40,000 in cephalin 0.15 per cent; 0.1 ml. adsorbed beef plasma; 0.1 ml. test plasma diluted 1:50; 0.1 ml. $CaCl_2$-serum eluate 5.1.

Proconvertin Assay.—When proconvertin deficient plasma was used as a substrate to determine the proconvertin activity of a diluted test plasma or serum, it was demonstrated that our patient's plasma and Stuart serum had proconvertin activity approximately equal to that of normal plasma (Table VIII). The substrate material used was lyophilized plasma from Owren's patient with a deficiency of proconvertin.

TABLE VIII. PROCONVERTIN ASSAY

Normal plasma	25.2 sec.
J. L. plasma	20.2 sec.
Stuart factor deficient serum	26.1 sec.
0 per cent (diluting fluid alone)	40.8 sec.

Test system: 0.1 ml. thromboplastin; 0.1 ml. adsorbed beef plasma; 0.1 ml. proconvertin deficient plasma; 0.1 ml. test plasma diluted 1:10; 0.1 ml. $CaCl_2$ 0.02 M.

Stability.—Normal plasma and serum stored at room temperature for 20 hours were both able to correct the prolonged prothrombin (Quick) time of the patient's lyophilized plasma (Table IX).

The thromboplastin generation was also restored to normal by substitution of stored serum for patient's serum (Table IX).

Total Thromboplastin Activity.—When the thromboplastin generation test was continued for 14 minutes, essentially no thromboplastic activity was noted (Table IX).

TABLE IX. STABILITY STUDIES

STORED PLASMA	STORED SERUM	PATIENT'S PLASMA	SALINE	CLOTTING TIME (SEC.)
Quick Times				
		0.05	0.05	147.0
	0.05	0.05		18.4
0.05		0.05		19.6

Thromboplastin Generation Test (*Thromboplastin Activity*)

Patient's plasma	+	Normal stored serum	+	Normal platelets:	100 per cent at 4 minutes—normal
Patient's plasma	+	Patient's serum	+	Normal platelets:	<5 per cent at 14 minutes—abnormal

DISCUSSION

The congenital hemorrhagic disorders characterized by a prolonged prothrombin time have been defined as deficiencies of labile and stable factors. The data presented here, and elsewhere,[9] demonstrate that our patient with a congenital hemorrhagic disease has a coagulation deficiency which is corrected by normal plasma and serum but not by barium sulfate adsorbed plasma. Therefore, by definition the deficiency of this patient is one of a stable factor.

That our patient does not have a proconvertin deficiency, as defined by Owren, seems clear. This was demonstrated by the capacity of the proconvertin deficient plasma to correct the patient's prolonged one-stage and two-stage prothrombin determinations. The patient's plasma contained a normal amount of prothrombin and proconvertin as measured in specific assays.

The observed abnormalities in the thromboplastin generation test and the prolonged Russell viper venom (Stypven) time, with and without addition of cephalin or thromboplastin, are similar, however, to those reported by Hougie, Barrow, and Graham[6] as characteristic of Stuart factor deficiency.

The diagnosis of Stuart factor deficiency in our patient rests on direct evidence in that Stuart factor deficient serum does not correct the patient's Quick time, prothrombin conversion rate, thromboplastin generation test or Russell viper venom time.

Telfer, Denson, and Wright[17] report a stable factor deficiency with an abnormal thromboplastin generation but a normal Russell viper venom time. Later, however, Biggs[18] states that she obtained an abnormal Russell viper venom time in Telfer's case. In the case report by Biggs[18] the thromboplastin generation test was delayed but when lecithin was used with Russell viper venom, a relatively normal time was obtained. The review by Ackroyd[19] of factor VII deficiency case reports includes a wide variation of the prothrombin

consumption values. It would seem that the ultimate test for proper classification would be that suggested by Hougie and co-workers of mutual exchange and cross-matching of plasmas and sera and testing in several clotting systems.

Hougie's patient was reported to have an abnormal prothrombin utilization (46 per cent remaining at one hour). The prothrombin utilization in our patient was normal, as measured by the two-stage prothrombin determination, and this result is in agreement with results previously reported on this patient by Frick and Hagen.[9] When prothrombin consumption was measured by the Russell viper venom-cephalin prothrombin method, consumption appeared to be at the lower limits of the normal range.

The abnormal thromboplastin generation and the normal prothrombin consumption appear incongruous since both tests depend upon the normal formation of plasma thromboplastin. However, the thromboplastin generation test is believed to be a more sensitive indicator of thromboplastin formation than is the prothrombin consumption test. The differences in these tests may indicate a quantitative, rather than a qualitative, difference in the activity of plasma thromboplastin in this patient.

Duckert and associates[20] have presented evidence for the existence of factor X. They have shown that factor X deficiency (1 per cent or more) limits the rate of thromboplastin formation but not total amount of thromboplastin. They also observed that factor X was destroyed by storage at room temperature for several hours. That our patient does not have a deficiency of factor X is suggested by the following: (1) ability of stored serum and/or plasma (20 hours at room temperature) to correct the abnormal prothrombin and thromboplastin generation tests of our patient, (2) the abnormal generation of thromboplastin of our patient's plasma demonstrates that there is not a limitation in the rate of development of thromboplastin, but rather that there is an inability to produce significant thromboplastic activity.

The Russell viper venom-cephalin method, as originally described, is independent of the level in the test plasma of proconvertin. The behavior of the patient's plasma was remarkably different from that of proconvertin deficient plasma. When an eluate from the barium sulfate adsorbate from normal serum was introduced as a reagent, the activity of the patient's plasma became normal, and presumably only prothrombin was measured. Therefore, the combination of Russell viper venom and cephalin, as well as the combination of Russell viper venom and thromboplastin, appears to require, for optimal activity, the presence of a stable, barium sulfate adsorbable factor present in normal plasma and serum which is different from the factor defined by Owren as proconvertin. These same combinations of thromboplastic agents do not require proconvertin for their optimal activity. This factor which is deficient in our patient is not identical with proconvertin, and may be considered identical with Stuart factor.

SUMMARY

1. A patient (J. L.) with a coagulation deficiency first reported as idiopathic hypoprothrombinemia and later as a deficiency of stable prothrombin conversion

factor, was restudied. Her deficiency appeared to be the same as that defined as Stuart factor deficiency.

2. The patient repeatedly demonstrated a prolonged Quick time, which was corrected by plasma from Owren's patient with proconvertin deficiency but not by serum from Hougie's patient with Stuart factor deficiency.

3. The two-stage prothrombin determination demonstrated a normal amount of prothrombin but a delayed rate of prothrombin conversion. This was corrected by normal plasma, normal serum, and proconvertin deficient plasma, but it was not corrected by normal barium sulfate adsorbed plasma or Stuart factor deficient serum.

4. The patient's abnormal thromboplastin generation test was corrected by normal serum and by hemophilia B serum, but it was not corrected by normal barium sulfate adsorbed plasma or Stuart factor deficient serum.

5. The patient's prolonged Russell viper venom (Stypven) time was corrected by normal plasma and by proconvertin deficient plasma, but not by Stuart factor deficient serum.

6. In the specific assay for proconvertin, the patient's plasma and Stuart's serum were observed to have normal activity. In a modified specific assay for prothrombin, using Russell viper venom and cephalin as thromboplastin and a serum eluate, the prothrombin activity was normal.

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COMPARATIVE ERYTHROPOIETIC INCORPORATION OF FERRIC AND FERROUS PREPARATIONS OF IRON-59 BY THE MONKEY, MACACA MULATTA

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RADIOISOTOPES have provided an ingenious key to the problem of following biochemical interrelationships in an organism. Iron-59 studies have revealed considerable information on hemoglobin metabolism, red blood cell formation, and blood dyserasias.

Radioiron is commercially available in either the ferric or ferrous state in concentrations of high specific activity. The literature does not advocate any one ionic form for intravenous injection. Either the ferric or ferrous ionic form will be incorporated into the red blood cell during erythrogenesis. However, when per cent incorporation of the ionic forms are compared, differences may become apparent.

To our knowledge no information is currently available concerning iron uptake in the monkey. This study was designed to compare degrees of incorporation of the two ionic forms of iron—ferric and ferrous—into monkey erythrocytes and compares this parameter with similar data obtained from other mammals.

MATERIALS AND METHODS

Twenty male *Macaca mulatta* monkeys with an age range of 4 to 6 years were selected for the study. Each animal was housed in a separate metal cage and maintained on a basic cereal ration supplemented with commercial vitamin and mineral mixtures. The animals had not been subjected to any other type of experimentation; however, one year prior to the initiation of this experiment, they inadvertently had been fed for 11 weeks a diet which contained an abnormally high concentration of vitamin D.[1] By the time of injection of the iron-59 preparations, all 20 animals appeared normal.

Both radioactive ferric chloride and ferrous citrate were purchased commercially.* Ferric-59 chloride was prepared for injection by mixing 1 ml. of the original sample (specific activity: 2040/mc./Gm. Fe) with 9 ml. physiologic saline.[2] The pH of the resultant solution was 3.5 The ferrous citrate was prepared for injection by mixing 1 ml. of the original sample (specific activity: 1925 mc./Gm. Fe) with 9 ml. sterile distilled water.[3] The pH of the solution was 6.5. Injection of the radioactive isotope was accomplished by drawing a 15 ml. sample of whole blood, to which 5 μc of the iron preparation was added. The syringe was

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*Ferric chloride and ferrous citrate were purchased from Abbott Laboratories, Chicago, Ill.

rotated gently for 30 seconds, then incubated at 37° C. A counting sample was removed from the syringe and the remaining blood-isotope mixture was injected into a femoral vein of the same animal from which the blood was drawn. Ferric chloride uptake was followed in two subgroups of five monkeys each. Injection of the two subgroups was carried out one month apart to test replication of results. Ferrous citrate uptake was followed in a single group of 10 animals one month after commencing the second ferric chloride group.

In subsequent discussion, the ferric-59 chloride preparation will be referred to as ferric-59, and that from the ferrous-59 citrate preparation will be referred to as ferrous-59.

Beginning 24 hours after injection of the radioactive iron and on specified days thereafter, a 4.5 ml. blood sample was withdrawn from each animal. A 1.0 ml. aliquot was reserved for accomplishing R.B.C. and W.B.C. counts and hemoglobin and hematocrit values. Standard laboratory procedures were employed.[4]

The remaining 3.5 ml. sample was analyzed for per cent incorporation of injected iron-59 into red blood cells in a Nancy Wood scintillation counter (model SC-2L). Blood samples were prepared for counting by spinning the red blood cells down at 2,000 rpm for 10 minutes and removing the plasma. The cells were washed once with physiologic saline, spun down again, and then washed into a polyethylene counting tube with tap water. The total volume in each counting tube was then 5 ml. The per cent incorporation of iron-59 into new red blood cells was calculated by the formula described by Hennessy and Huff.[5] In our calculations of blood volume for the monkey, the figure of 60.9 ml./kg. body weight was used.[6]

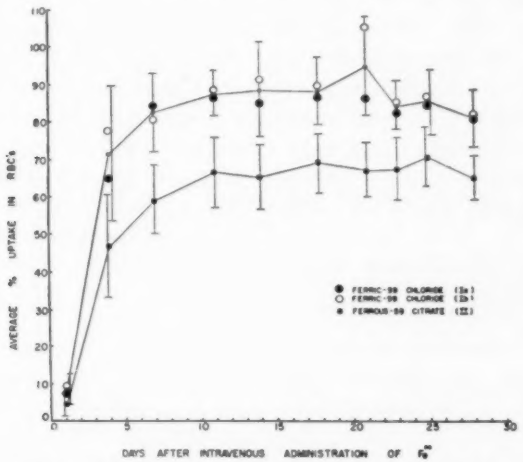
AVERAGE % UPTAKE IN RBC's
DAYS AFTER INTRAVENOUS ADMINISTRATION OF Fe59
FERRIC-59 CHLORIDE (In)
FERRIC-59 CHLORIDE (Dr)
FERROUS-59 CITRATE (□)

Fig. 1.—Comparative average per cent uptake ± s.d. of ferric-59 chloride and ferrous-59 citrate in the R.B.C. of the monkey, *Macca mulatta*.

RESULTS

Fig. 1 reflects the per cent of injected radioiron which was incorporated into new erythrocytes in the two subgroups which received ferric-59 and in the ferrous-59 group. Since the two ferric-59 subgroups exhibited essentially the same uptake curves, the line representing the ferric-59 group was obtained by averaging all ferric-59 animals sampled on a particular day postinjection. Average per cent incorporation for the ferric-59 animals was consistently greater than for the ferrous-59 group.

On subjecting the data to the "t" test, it was found that differences between the ferric and ferrous groups were significant to the 0.05 level on the first day postinjection and significant to the 0.01 level on all subsequent days (Table I).

TABLE I. COMPARATIVE IRON UPTAKE

DAYS POST-INJECTION OF Fe^{59}	PER CENT UPTAKE				SIGNIFICANCE	
	+++ $FeCl_3$ (10 ANIMALS)		++ FE CITRATE (10 ANIMALS)			
	AVERAGE PER CENT	S.D.	AVERAGE PER CENT	S.D.	"t" TEST	MANN-WHITNEY U TEST
1	8.3	4.0	4.6	3.1	<0.05	<0.1
4	71.3	18.0	46.8	13.6	<0.01	<0.01
7	82.2	10.5	59.0	8.8	<0.01	<0.01
11	87.2	6.1	66.2	9.5	<0.01	<0.01
14	97.9	12.6	64.8	8.9	<0.01	<0.01
18	87.5	9.2	68.3	7.9	<0.01	<0.01
21	93.9	12.9	66.5	7.2	<0.01	<0.01
23	83.6	6.5	66.7	8.5	<0.01	<0.01
25	84.1	8.2	70.0	7.9	<0.01	<0.01
28	80.1	7.2	64.4	5.7	<0.01	<0.01

The data was also subjected to the Mann-Whitney U test.[7] This would test the data on a rank distribution rather than an average mean uptake. For each of the 10 days on which samples were secured, the uptake measures of samples in both groups were ranked together, as for a sum of rank tests. Then, in order to increase the stability of ranking by considering all 10 measures on each animal, a total of ranks assigned to each sample in the 10 separate rankings was calculated. The total ranks were then ranked to yield one combined ranking of independent measures, and the Mann-Whitney U test was employed to test daily differences and the over-all significance of the difference between the groups. Separation on the first day after injection was significant to the 0.1 level, but on all subsequent days the groups were separated to the 0.01 level of significance (Table I). Separation between the two groups, over all days, was found significant to beyond the 0.002 level.

Both groups of animals had approximated an incorporation asymptote by day 11. The mean value and its standard deviation for the plateau (days 11 through 28) gave an amplitude value of 86.3 ± 4.4 per cent for the ferric^{-59} group. Mean per cent incorporation for the ferrous^{-59} group had the dimensions of 66.7 ± 1.9 per cent during the same period. Differences in mean amplitudes of incorporation in the ferric^{-59} and ferrous^{-59} groups were found to be significant to the 0.01 confidence level.

Because of the sampling frequency, it was necessary to concurrently observe the peripheral blood picture in all animals to determine the effects of sampling. Erythrocyte and white blood cell counts, as well as hemoglobin and hematocrit values were followed. Analysis of the data did not reveal any appreciable effect.

DISCUSSION

Evaluation of the iron uptake data by both the "t" test and the Mann-Whitney U test indicated a significant difference between the plateaus of ferric^{-59}

and ferrous^{-59}. This study encompassed no attempt to evaluate the effects of the anionic component of the compounds injected. However, the results suggest that either the ionic state of the iron, its anionic component, or the method of preparing the compound for injection may influence the amount of radioiron which is incorporated by the erythropoietic system of the monkey.

The literature reveals that many dynamic values, such as erythropoietic activity and red cell iron turnover, are based on either rate or total incorporation of radioiron by the organism. It is equally apparent that several radioiron compounds are in general use by investigators, and that modes of preparation vary. It is suggested by this study that considerable care should be exercised in striking comparisons between the data accrued by different investigators.

The per cent of ferric^{-59} uptake in the male *Macaca mulatta* monkey compares favorably with human uptake values. Using a ferric chloride solution, Badenbaek and Callender[8] reported an approximate average in human beings of 90 per cent uptake in one week. For monkeys, the 7-day uptake value was found to be 82 per cent. Bothwell and associates[9] reported an average human red cell incorporation of 90 per cent of injected ferric^{-59} chloride.

SUMMARY

1. Twenty male *Macaca mulatta* monkeys were divided into two equal groups. One group of 10 animals received 5 μc ferrous^{-59} citrate per animal. The other 10 animals were subdivided into 2 groups of 5 animals each. Each animal received 5 μc ferric^{-59} chloride, but injections in the two subgroups were separated by a temporal lapse of one month. Animals in both primary groups received intravenous injections of their own blood after it had been incubated with the radioiron compound.

2. The ferric chloride preparation was incorporated to a significantly greater degree than was the ferrous citrate preparation. The contributing factors to this difference—cationic state, anionic state, pH of preparation—have not been tested separately.

3. Concurrent analysis of the peripheral blood picture during the period of sampling indicated no appreciable deleterious effects attributable to injection or sampling.

We wish to acknowledge the assistance of Robert Riess and James Davis in the collection of the data.

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THE UTILIZATION OF SACCHARATED Fe^{59} OXIDE IN RED CELL FORMATION

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INTRAVENOUS injection of saccharated iron oxide is a highly effective treatment for the anemia of iron deficiency. It is generally assumed that this material is completely utilizable for hemoglobin formation in iron deficient patients.[1] Several independent observers have noted that the bone marrow of patients who develop relapses of iron deficiency anemia after intravenous saccharated iron oxide therapy contains stainable iron granules.[2-4] In the present studies, the utilization of intravenously administered Fe^{59}-labeled saccharated iron oxide for red cell formation has been measured. This has been done for two reasons: (1) to determine whether utilization of saccharated iron oxide is as complete in iron deficient subjects as is generally assumed; and (2) to determine whether this material may be used as a practical means of measuring the turnover and presumably the size of iron stores[5] since it appears as stainable deposits in reticulum cells very soon after injection[6] and might, therefore, be expected to behave like storage iron. Preliminary observations[7] in normal and iron deficient rats revealed a moderate difference in the incorporation of radioactive saccharated iron oxide into the red cells. Clinical studies therefore appeared justified.

MATERIALS AND METHODS

Fe^{59}-labeled saccharated iron oxide was obtained from Abbott Laboratories, and prepared according to the method of Nissim and Robson.[8] The labeled saccharated iron oxide was diluted appropriately with physiologic saline and with commercially available saccharated iron oxide† to give an iron concentration of 2.50 mg. per ml. and radioactivity of 0.5 to 1.3 μc per ml.

Ten patients were studied. The pertinent clinical data are given in Table I. Evaluation of the iron stores by sternal puncture and the plasma iron and iron binding capacity determinations were carried out as described previously.[4] Saccharated iron oxide solution was given intravenously in a dose of 0.1 ml. per kilogram of body weight, or 0.25 mg. per kilogram. The radioactivity ranged from 0.05 to 0.13 μc per kilogram. No untoward effect was noted in any patient.

Ten milliliter blood samples were withdrawn at suitable intervals and were counted in a well-type scintillation counter. The blood volume has been assumed to be 7 per cent of the body weight, and the results have been expressed as the percentage of administered radioactivity appearing in the peripheral blood.

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†Proferrin; Sharp & Dohme, Inc. (Merck Sharp & Dohme), Philadelphia, Pa.

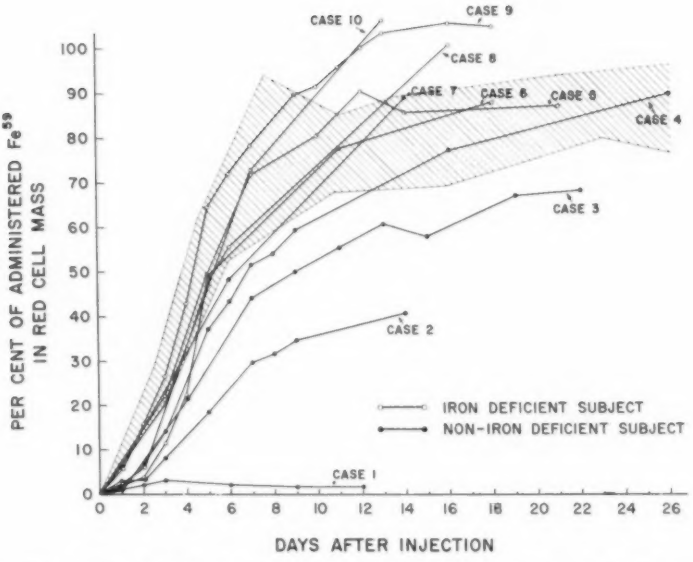

Fig. 1.—The uptake of iron from saccharated Fe^{59} oxide into red cells in 10 patients. Pertinent clinical data are presented in Table 1. Patients with depleted iron stores are regarded as "iron deficient." The shaded area represents the range of normal incorporation given by Moore and associates[9] for plasma-bound iron.

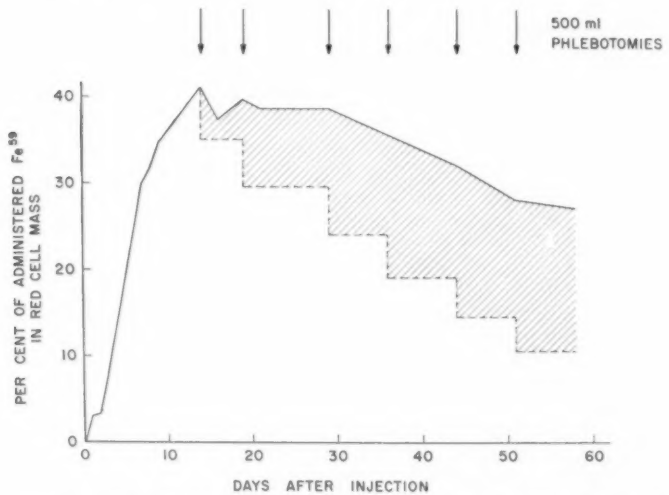

Fig. 2.—The uptake of iron from saccharated Fe^{59} oxide into the red cells of a patient with idiopathic hemochromatosis. The broken line indicates the expected level of radioactivity in the blood had no Fe^{59} entered the red cells after the fourteenth day. The shaded area, therefore, represents iron mobilized after that day.

TABLE I. CLINICAL DATA

CASE NO.	SEX	DIAGNOSIS	HEMOGLOBIN (GM. %)	PL. IRON γ/100 ML.	UIBC* γ/100 ML.	BONE MARROW IRON	COMMENT
1	F	Acute leukemia	9.4			+ +	
2	F	Idiopathic hemochromatosis	13.0	240	0		6,000 ml. of blood had already been withdrawn (none for 3 weeks). After 12,000 ml. more were removed, the readily mobilized stores were exhausted
3	M	Inactive rheumatic heart disease	16.3				
4	F	No organic disease; slight malnutrition	13.1	50	160	+ +	
5	F	Iron deficiency anemia	11.9	25	375	0	
6	F	Excessive fatigue	12.7	46	265	0	
7	F	No organic disease	12.7	107	160	+ +	
8	F	Excessive fatigue	13.5	70	200	0	
9	F	Relapse of iron deficiency anemia	10.0	5	360	+ +	Treatment with saccharated Fe^{59} oxide 4 months previously
10	F	Iron deficiency anemia	7.9	7	470	0	

*UIBC = Unsaturated iron binding capacity.

RESULTS

The results of the clinical studies are presented graphically in Fig. 1. The utilization of the saccharated iron oxide for red cell formation was quite comparable to that of plasma-labeled iron, as has been reported by others.[9] Although it is apparent that the incorporation of iron was more rapid and more complete in iron-depleted subjects than in noniron-depleted subjects, the difference is not large. A patient (Case 9) whose marrow showed the presence of stainable iron following relapse of iron deficiency anemia after treatment with saccharated iron oxide utilized the labeled saccharated iron oxide very well. The patient with hemochromatosis (Case 2) showed a reduced incorporation of Fe^{59} into the red cells. Fig. 2 shows the Fe^{59} levels in the blood of this patient following a series of phlebotomies. If none of the saccharated Fe^{59} oxide had been mobilized, a greater decrease in radioactivity than was observed would have occurred.

DISCUSSION

Although the utilization and turnover of tracer amounts of plasma-bound radioiron have been studied extensively, relatively little attention has been paid

to the fate of other iron compounds introduced into the body. The fate of such compounds is of particular interest because, theoretically, it offers a means for the measurement of iron stores. Excellent differentiation between iron-deficient and normal subjects has been reported by Finch and associates,[10] using devitalized red cells to tag the iron storage compartment. This method of measurement has very limited practical application, however, because of the risk (e.g., hepatitis and sensitization) of introducing foreign red cells into the circulation and because of the difficulty in procuring human Fe^{59}-labeled red cells. Studies by Künkel and associates[11] using "ferronasein" and by Garby and Sjölin[12] using iron-dextran, have shown some difference between utilization of these compounds by iron deficient and normal subjects, but the difference has been quite small when compared with that observed by Finch and his co-workers[10] with devitalized red cells.

The appearance of Fe^{59}-labeled saccharated iron oxide in red cells has not, to our knowledge, been studied previously, except by nonquantitative (autoradiographic) means.[13] The present study indicates that although differentiation between iron deficient and noniron deficient subjects is somewhat better using saccharated iron oxide than plasma-bound iron, the differentiation does not appear sufficiently clear to be of real clinical value. Moreover, it is possible that at least part of the difference observed may be more apparent than real, since the assumption of a normal blood volume in anemic patients may represent an overestimate. Such an overestimate would tend to exaggerate the calculated uptake of iron into the red cells of patients with iron deficiency anemia.

The excellent utilization of the labeled saccharated iron oxide for blood formation appears to confirm the clinical impression that this iron is readily available for erythropoiesis. It is necessary, however, to reconcile the efficient utilization of radioiron in our subjects with the finding that stainable iron persists in the marrow after the relapse of iron deficient patients treated with saccharated iron oxide.[2-4] It is possible that the excellent utilization of saccharated iron oxide, as observed in this study, occurs only when small dosages are given. Iron that is not used immediately for erythropoiesis may spill over into secondary receptors that less readily give up their iron for erythropoiesis. While storage mechanisms in hemochromatosis admittedly may not be normal, this concept gains some support from our study of the rate of appearance of radioiron in the blood of a subject with hemochromatosis undergoing phlebotomy. While in the first 7 days 30 per cent of the administered dose appeared in the peripheral blood, only 10 per cent more appeared in the subsequent 7 days. In the succeeding 44 days, even under the stress of repeated phlebotomy, only about 18 per cent of the administered radioiron was utilized for erythropoiesis.

SUMMARY

1. Saccharated Fe^{59} oxide has been administered intravenously to 10 patients.

2. Utilization of Fe^{59} for red cell formation was rapid both in iron deficient and nondeficient subjects.

3. Uptake into the red cells of iron deficient patients was somewhat more rapid than uptake into the red cells of individuals with normal iron stores. This difference was not sufficiently great, however, to be of practical value in evaluating the size of iron stores.

4. It appeared possible that when larger doses of saccharated iron oxide than those used in this study are given, that iron not used immediately for erythropoiesis spills into secondary receptors from which it is extracted with difficulty. This would explain the finding of stainable iron deposits in the bone marrow of iron deficient patients who have been treated with saccharated iron oxide and have subsequently relapsed, a finding which otherwise appears inconsistent with the excellent utilization of this compound by iron deficient subjects.

5. This speculation has been shown to gain some support from measurement of the utilization saccharated Fe^{59} oxide in a subject with hemochromatosis undergoing frequent phlebotomy.

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EFFECT OF SERA FROM PATIENTS WITH ANOXIA ON THE PROLIFERATIVE ACTIVITY OF HUMAN ERYTHROPOIETIC TISSUE IN VITRO

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THE importance of hypoxia in the homeostatic control of erythropoiesis is generally recognized. The manner in which the hypoxic stimulus to erythropoiesis operates is, however, not completely understood. No hypoxia of the bone marrow itself could be demonstrated in anemia.[1, 2] Furthermore, it was shown in bone marrow cultures that low concentrations of oxygen decreased, rather than increased, the proliferative activity of erythroid cells.[3, 4]

Carnot and Deflandre's[5] suggestion of a circulating "hématopoietine" has aroused much interest in this humoral factor mediating the anoxic stimulus. Most subsequent workers have used Carnot and Deflandre's original experiment as a model. They injected plasma or plasma fractions from animals made anoxic by various means into normal animals and studied its effect on erythropoiesis in the recipient animals. Methods employed by various workers in this field have been open to criticism. In addition, there has been no agreement on the criteria to be applied in evaluating the evidence for an effect of hematopoietine on the peripheral blood. Results, therefore, have been conflicting. Although recent work[6-8] has added considerably to the weight of evidence for a humoral anoxic factor stimulating erythropoiesis, it has not yet been universally accepted.

In the present investigation the tissue culture technique was applied to study the activity of the humoral anoxic factor. It was felt that an in vitro system, in which the immediate effect of various factors on the erythropoietic tissue itself could be observed, offered a more direct approach to the problem.

The system employed consisted of normal human bone marrow cell suspensions, incubated in homologous plasma clots, in the presence of normal serum and of sera from patients with various forms of anoxia. Changes in the number of erythroid cells capable of mitosis (i.e., early and intermediate normoblasts) in cultures with various sera were followed quantitatively.

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MATERIALS AND METHODS

1. Bone marrow. Normal bone marrow was obtained by spinous process aspiration from patients with no obvious hematologic disorders and collected into siliconed test tubes. Needles and syringes were previously rinsed with heparin.

Bone marrow was preferably obtained from group O individuals, or else only sera compatible with the bone marrow in respect to the major blood groups were used in the cultures.

2. Heparin solution* containing 10 mg./ml.

3. Gey's solution[9] was Seitz filtered, adjusted to pH 7.4 and kept in the frozen state in rubber-stoppered flasks.

4. Plasma. Normal plasma, freshly obtained, of blood group compatible with the bone marrow. Five milliliters of blood were drawn into 0.5 ml. of heparin solution diluted 1:30 in Gey's solution to give a final concentration of heparin of 33γ/ml.

5. Thrombin solution. Bovine thrombin* dissolved in 5 ml. Gey's solution to give a concentration of 200 units/ml.

6. Serum. Normal serum was obtained from group AB blood bank donors. The serum was separated under aseptic conditions, divided into small portions and kept in the frozen state. The same normal serum was used throughout the phase of the work covered by the present report.

Pathologic sera were obtained (aseptically) and kept in the frozen state for varying periods of time, from a few days to several weeks.

7. Penicillin and Streptomycin were added to the culture medium to a final concentration of 200 units and 200γ/ml., respectively.

8. Trypsin† activity 1:250, 2 per cent solution.

9. Glassware was cleaned according to methods used in tissue culture work. Pyrex tubes were used for cultures. All other glassware with which the bone marrow cells came in contact, including syringes, were siliconed.

The bone marrow aspirate was washed twice in Gey's solution. To the packed washed cells approximately half their volume of thrombin solution was added and the cells resuspended by gentle agitation for 2 minutes.

One-tenth milliliter aliquots of plasma were measured into the bottom of culture tubes, using 0.1 ml. blow-out pipettes and spread over the side of the tube. In the same manner aliquots of 0.1 ml. of the bone marrow cells suspended in thrombin solution were then introduced into the culture tubes and spread over the plasma layer. The tubes were held in a horizontal position until a firm clot had formed.

The tube containing the bone marrow suspension was repeatedly agitated while material was withdrawn from it for introduction into culture tubes. Before and after withdrawal two aliquots of 0.1 ml. were taken from the bone marrow suspension and introduced each into a tube containing 2 ml. of Gey's solution for an initial total nucleated cell count.

Two culture tubes were put aside and the clots which had formed in them used for the preparation of initial smears. To each of the remaining tubes, 1 ml. of fluid medium was then added. The fluid medium consisted of 50 to 75 per cent serum to be tested in Gey's solution. The culture tubes were divided into sets of 3. Fluid medium containing the same serum was added to each set of 3 tubes.

The culture tubes were then rubber stoppered and incubated in a roller apparatus at 37° C. for 17 hours.

At the end of incubation 1 ml. trypsin solution was added to each of 2 tubes of each set of 3 containing the same serum. A total nucleated cell count in duplicate was done on the trypsinized material in these tubes. With the addition of trypsin the total volume of fluid in each tube was brought up to 2.2 ml., making the final dilution of the bone marrow

*The Upjohn Company, Kalamazoo, Mich.
†Difco Laboratories, Detroit, Mich.

cell suspension 1:22. Counts were done with a white cell counting pipette by drawing glacial acetic acid up to the 0.5 mark and filling the pipette up to the 11 mark with cell suspension. From the intact pellicle of clotted plasma in the third tube of each set, imprint smears were prepared for postincubation differential counts. Differential counts done on these smears were comparable to those done on the initial smears, prepared in similar fashion. The smears were stained with May-Grünwald-Giemsa.

From the mean of total counts in duplicate tubes and the differential counts absolute numbers of early and intermediate normoblasts were calculated. Individual total counts agreed to within ± 5 per cent of the mean. Differential counts were done on 3 to 5,000 cells.

RESULTS

Cellular elements found in 17-hour cultures were morphologically normal and represented all stages of development of the red, white, and megakaryocytic series. The quantitative estimation of proliferative activity in the red series, in the presence of normal and "anoxic" sera, is summarized in Table I.

The number of erythroid cells capable of mitosis (early and intermediate normoblasts) found after incubation of a given bone marrow cell suspension with various sera was considered to reflect the proliferative activity of that cell population as modified by the respective sera. As conditions in culture tubes varied only in respect to the serum added, loss of early and intermediate normoblasts due to maturation and cell death was assumed to be of the same order of magnitude in all cultures. Differences in numbers of these cells present after incubation could therefore be accounted for by differences in the number of cells added during incubation. No separate figures are given for early normoblasts. Although these cells followed the general trend, they did not, because of their small numbers, form a basis for calculation. They were, therefore, counted together with intermediate normoblasts. The relatively small proportion of early and intermediate normoblasts in the general population of nucleated cells is explained by dilution with peripheral blood, which was unavoidable during aspiration of the large amounts of bone marrow material required for this work. Data in Table I are arranged in blocks representing individual experiments and comprising groups of pathologic sera preceded by the normal serum with which they were tested. Within each experiment the number of cells present before incubation is used as a standard of reference for percentage changes during incubation. For comparison of results between experiments, values found after incubation with pathologic sera are expressed as percentages of that value found with normal serum in the respective experiment.

The number of early and intermediate normoblasts found after 17 hours' incubation in the presence of normal serum is about equal to the number before incubation. This is taken as evidence of cell proliferation which has occurred in cultures with normal serum. Had this not been the case, maturation to later forms and cell death would have been expected to cause a sharp drop in the number of earlier forms. Enough cell proliferation to balance this cell loss must, therefore, have taken place in the presence of normal serum under the conditions of our experiments.

Sixteen anoxic sera were examined. They included 4 sera from patients with polycythemia secondary to cyanotic congenital heart disease and 12 sera from patients with anemia of varying etiology.

Four sera were examined each on 2 occasions. Data obtained with these sera in different experiments are brought together in Table II for comparison. Correlation of results, expressed as percentages of the normal control used in each experiment, is very good in 3 repeat experiments and fair in one. As the same normal serum was used in all experiments, the reproducibility of results may further be judged by comparing the results obtained with normal serum in different experiments. These ranged between 87 and 117 per cent of preincubation values.

Results were arbitrarily divided into 3 groups:

1. An increase in erythroid cells below 130 per cent of the control serum in the respective experiment was considered not significant.

2. An increase of 130 to 180 per cent was considered significant.

3. An increase of over 180 per cent was considered highly significant.

Highly significant increases over the normal controls were obtained with 6 sera. Significant results were obtained with 7 sera (out of 2 sera in this group examined twice, results below the limit of significance were obtained with one serum on repeat examinations). No significant increase in erythroid cells over the control serum was observed with 3 sera.

Considered as a whole, therefore, our data provide strong statistical evidence for a humoral anoxic factor stimulating erythropoiesis. In the following, analysis of results in individual cases will be attempted.

Of the 4 sera from patients with cyanotic congenital heart disease with polycythemia, 2 sera stimulated erythopoiesis in vitro to a degree roughly proportionate to the degree of polycythemia which had existed in vivo.

Case 1. I. B. was a 13-year-old boy with a hematocrit of 90 per cent and extremely severe cyanosis, probably due to transposition of the great vessels. His serum gave an increase in erythropoiesis of 267 and 224 per cent, respectively, on 2 occasions.

Case 11. Z. N. was a 6-month-old girl, a probable case of tetralogy of Fallot, with an as yet mild cyanosis and polycythemia (R.B.C. 6 million, hemoglobin 16 Gm. per cent). Her serum, examined twice, showed a mild degree of stimulation of erythropoiesis (144 and 130 per cent, respectively).

Case 14. D. H. was a 2-year-old boy diagnosed as having tetralogy of Fallot. He probably had associated iron deficiency, as evidenced by the hypochromic blood picture (R.B.C. 7.3 million, hemoglobin 14.1 Gm. per cent). Blood formation was limited by lack of iron so that the powerful stimulation of erythropoiesis (233 per cent) by the boy's serum was probably a better index of the anoxia which existed in vivo than was the patient's hemoglobin level.

A stimulation below that expected from the degree of polycythemia was found with the serum of J. R. (Case 6).

TABLE I. CHANGES IN EARLY AND INTERMEDIATE NORMOBLASTS FOLLOWING INCUBATION WITH NORMAL AND ANOXIC SERA

SERUM USED	INCUBATION (HOURS)	PER CENT SERUM IN MEDIUM	TOTAL N.C. PER C.MM.	E + I PER C.MM.	E + I PER CENT PREINCUBATION	E + I PER CENT NORMAL
	0		47,550	2,495	100	
Normal	17	50	49,150	2,160	87	100
Case 1, J. B.; C. c. heart disease, Hb. 25, R.B.C. 10 million	17	50	48,700	5,770	231	267
Case 2, S. G.; Thalassemia major, Hb. 5.4	17	50	46,000	2,852	114	132
	0		46,000	2,116	100	
Normal	17	50	44,700	1,993	95	100
Case 1, J. B.; C. c. heart disease, Hb. 25, R.B.C. 10 million	17	50	52,000	4,472	213	224
Case 3, L. R.; Ac. leukemia, Hb. 7.2, R.B.C. 2.5 million	17	50	45,800	1,534	73	77
Case 4, B. T.; Ac. leukemia, Hb. 7.2, R.B.C. 2.5 million	17	50	45,000	2,250	107	112
	0		57,000	6,327	100	
Normal	17	50	56,800	6,099	97	100
Case 5, J. L.; Thalassemia major, post splenectomy, Hb. 5.5, Retics. 6.7 per cent	17	50	64,000	5,504	87	90
Case 6, J. R.; C. c. heart disease, Hb. 21.3, R.B.C. 7.4 million	17	50	66,400	8,976	142	146
	0		41,300	1,980	100	
Normal	17	75	47,400	2,068	104	100
Case 7, S. H. R.; Hered. spherocytosis aplast. crisis, Hb. 2.8, R.B.C. 0.94 million, Retics. 0	17	75	40,100	3,680	186	180
Case 8, D. R.; S. H. R.'s brother; hered. spherocytosis aplast. crisis, Hb. 4.0, R.B.C. 1.4 million, Retics. 0	17	75	49,400	3,950	200	190

TABLE I—CONT'D

Case 9, N. S.; Favism, Hb. 3.4, R.B.C. 1.37 million, Retics. 31 per cent	17	75	49,400	3,500	175	170
Case 10, J. B. H.; Hypochromic anemia, Hb. 3.1	17	75	45,000	4,050	204	195
Case 11, Z. M.; Tetralogy of Fallot, Hb. 16.0, R.B.C. 6 million	17	75	45,600	2,763	140	133
Case 12, J. A. H.; Favism, Hb. 5.3, R.B.C. 1.75 million, Retics. 14 per cent	17	75	43,800	2,508	127	121
	0		21,700	630	100	
Normal	17	75	29,000	660	105	100
Case 13, J. N.; Thalassemia major, Hb. 3.5, R.B.C. 1.26 million, Retics. 2.8 per cent	17	75	28,300	1,190	189	180
Case 11, Z. M.; Tetralogy of Fallot, Hb. 16.0, R.B.C. 6 million	17	50	22,600	860	136	130
Case 14, D. H.; C. c. heart disease, Hb. 14.1, R.B.C. 7.3 million	17	75	29,100	1,540	245	233
	0		35,000	1,470	100	
Normal	17	50	37,700	1,734	117	100
Case 5, J. L.; Thalassemia major, post splenectomy, Hb. 5.5, Retics. 6.7 per cent	17	50	35,850	1,620	110	95
Case 15, A. L.; Periarteritis nodosa + acq. hemolytic anemia, Hb. 4.1, R.B.C. 1.42 million, Retics. 15 per cent	17	50	35,250	2,485	170	146
Case 16, Ar. L.; Thalassemia major, J. L.'s brother, Hb. 4.2, Retics. 6.9 per cent	17	50	37,900	2,698	183	158
Case 4, B. T.; Ac. leukemia, Hb. 7.2, R.B.C. 2.5 million	17	50	38,000	2,681	182	157

N.C. = nucleated cells; E + I = sum of early and intermediate normoblasts.

TABLE II. VARIABILITY OF RESULTS OBTAINED WITH ANOXIC SERA EXAMINED ON 2 OCCASIONS, EXPRESSED AS PERCENTAGES OF THE NORMAL SERUM IN EACH EXPERIMENT

CASE	PER CENT OF NORMAL	
	1	2
1. J. B.	267	224
4. B. T.	157	112
5. J. L.	95	90
11. Z. M.	133	130

The group of anemias comprised 12 sera. Of 7 sera obtained from patients whose hemoglobin level was below 5 Gm. per cent, 4 gave highly significant and 3 gave significant increases in erythropoiesis over the normal. In this group the R. brothers (Cases 7 and 8) are of interest. Both were proved cases of hereditary spherocytosis. They were seen during an aplastic crisis which they developed simultaneously following an acute infection, probably of viral origin. S. H. R.'s hemoglobin was down to 2.8 Gm. per cent when the serum was obtained, while D. R.'s hemoglobin level was 4.0 Gm. per cent. No reticulocytes were found and serum bilirubin was low. In vitro, powerful stimulation of erythropoiesis was obtained with these patients' sera.

No significant stimulation was observed with 3 sera from patients whose hemoglobin level was above 5 Gm. per cent. Included in this group are sera from 2 cases of acute leukemia with relatively mild anemia (Cases 3 and 4), one of which showed some degree of stimulation on repeat examination. A case of thalassemia major after splenectomy (Case 5), in spite of a low hemoglobin level of 5.5 Gm. per cent, gave results slightly below the normal control on repeat examination.

DISCUSSION

An in vitro approach to the problem of the humoral anoxic stimulus to erythropoiesis offers the following obvious advantages:

1. With suitable technique of bone marrow culture, changes in the erythropoietic tissue itself can be followed quantitatively.

2. Amounts of serum or plasma required are far smaller than those needed for injection into animals.

3. Human material can be studied in a homologous system.

The methods proposed for the cultivation of bone marrow can be classified as falling into one of 2 groups. The first group comprises methods based on the classical technique of tissue culture in a plasma clot. While active cell proliferation does take place in plasma clot cultures, the method does not lend itself to quantitative work. The second group consists of various modifications of the technique of bone marrow culture in a fluid medium first proposed by Osgood.[10] The fluid medium technique has been extensively used by hematologists because of the ease with which cell counts can be done. However, there is practically no proliferative activity in fluid medium cultures. Using a fluid medium technique[11] we found cell proliferation to be equally low in cultures with anoxic and normal sera (unpublished data).

For the present study, therefore, a method has been evolved which combines features of both techniques. Culture in a plasma clot provided conditions favorable to cell proliferation, while accurate measurement of all components of the system and ultimate lysis of the clot with trypsin enabled quantitative estimation of the results. Two important departures from the "classical" technique were the omission of chicken plasma and the substitution of thrombin for the traditional embryonal extract. With this substitution the formation of firm clots could be better controlled and any excessive stimulation of proliferation by embryonal extract could be avoided.

Although results obtained with normal serum and several pathologic sera agreed fairly well in repeat experiments, no claim of accuracy is made for the method, relying as it does on figures derived indirectly by calculation from total and differential counts. Further refinement of the method is necessary before it can be used as a quantitative assay of anoxic factor activity. Yet the method was sensitive enough to demonstrate conclusively a significant increase in erythropoiesis in the type of case chosen for this study and representing *extreme* examples of anoxia.

The in vitro response depended on the degree of anoxia present in vivo, irrespective of its type, whether anoxic or anemic, or of the mechanism leading to anemia. Furthermore, anoxic factor activity, as demonstrated in vitro, did not necessarily parallel the response to the anoxic stimulus observed in vivo. In patients with congenital heart disease, possessing an intact erythropoietic apparatus, this stimulas expressed itself in producing higher hemoglobin levels. On the other hand, anemias, of whatever etiology, were characterized by a gap between the actual low level of hemoglobin and the correction of the anemia, which is the ultimate goal of the anoxic stimulus. The two brothers with hereditary spherocytosis during an aplastic crisis are good examples of a situation in which anoxic factor is present in the serum in amounts sufficient to stimulate intensive erythropoiesis in vitro, and yet in vivo this activity cannot be translated into higher cell counts, because it finds the bone marrow temporarily put out of action. A similarly low hemoglobin level in spite of a high erythropoiesis stimulating activity of the serum was found in the case of severe hypochromic anemia (Case 10). Here it is assumed that lack of iron was the cause of the inability of the bone marrow to respond. In the 3 examples of acquired hemolytic anemia in our series (favism, Cases 9 and 12, and acquired hemolytic anemia accompanying periarteritis nodosa, Case 15), the high reticulocyte count was good evidence of an in vivo response to the anoxic stimulus demonstrated in vitro.

A stimulation of erythropoiesis was obtained by sera from cases of thalassemia with low hemoglobin levels with the notable exception of the splenectomized patient (Case 5), whose serum, on 2 occasions, gave results slightly below the normal control. Further experience with splenectomized patients is needed before any speculation on the role of the spleen in the production of anoxic factor can be attempted.

No significant stimulation of erythropoiesis was obtained with sera of 2 patients with acute leukemia (Cases 3 and 4). Although the method may not be sensitive enough for the milder degree of anemia found in these 2 cases, the possibility of some interference with anoxic factor activity in acute leukemia leading to anemia deserves further study.

SUMMARY

A method for the culture of bone marrow cell suspensions is described. It provides conditions favorable for cell proliferation and allows changes in the cell population to be followed quantitatively.

Normal human bone marrow was grown, according to the method described, in the presence of sera from patients with anoxia. These comprised cases of severe anemia and of polycythemia secondary to cyanotic congenital heart disease.

A significant increase in the proliferative activity of erythroid cells grown with "anoxic" sera, as compared with normal serum, was observed. This increase depended on the degree of anoxia present in vivo, irrespective of the type, whether anemic or anoxic.

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A STUDY ON ORANGE-PIGMENTED NON-PHOTOCHROMOGENIC MYCOBACTERIA

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IT IS the purpose of this paper to record a study of cultural and pathogenic properties of 7 strains of orange-pigmented mycobacteria, isolated from man, the pigment of which is formed independent of light. There appears to be a good number of such isolates made from clinical material by diagnostic laboratories. Whether this is due to improved bacteriologic techniques, the result of a selective process created by the extensive use of chemotherapeutic agents, or whether these organisms are mutants induced by drugs is not known. It is a common practice among clinicians and bacteriologists to regard all pigmented mycobacteria as saprophytes. This is unfortunate especially in the light of recent findings.[1-4] An accurate classification of atypical mycobacteria can be made only if thorough bacteriologic and histopathologic examinations are performed.

Several investigators[1-13] have reported chromogenic mycobacteria in human tissue, sputum, nasopharynx, cerebrospinal fluid, gastric fluid, skin lesions, urine, and blood. Some attempts to characterize these organisms have been made by investigating their cultural, immunologic, and histopathologic properties.[14-20] However, they were unclassified until a survey on atypical mycobacteria was conducted by Runyon under the auspices of the Veterans Administration—National Tuberculosis Association[21] in 1955. From this study it was concluded that chromogens with light-sensitive pigment comprise a homogenous group because their cultural characteristics, sensitivity to antimicrobial agents, and pathogenicity for mice and/or hamsters are reasonably consistent. Hauduroy[22] proposed the name *Mycobacterium kansasii* for these organisms. The chromogenic mycobacteria, called scotochromogens, whose pigment is formed independently of light consists of a heterogenous group of organisms that is still unclassified. The strains being reported here belong to this latter group.

MATERIALS AND METHODS

Organisms.—Seven orange-pigmented mycobacteria were studied. They were isolated from patients on the tuberculosis observation service of Wayne County General Hospital, Eloise, Mich. The cases were not selected; the organisms were recovered during routine examinations made over a 3-month period. The following are brief histories of the patients from whom these organisms were isolated.

This study was made in the Department of Bacteriology of the University of Michigan Medical School, Ann Arbor, Mich., and submitted as partial fulfillment of the requirements for the Master of Science degree in bacteriology.

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Patient R. P. Chest x-ray in August, 1953, was strongly suggestive of tuberculosis. Skin test to 1:100 dilution of Old Tuberculin (O.T.) was positive. Two specimens of gastric fluid and 1 of sputum cultured between August and September, 1953, were negative; 11 x-rays between December, 1953, and July, 1955, failed to show evidence of active tuberculosis. The mycobacteria used in this study were isolated from sputum in March, 1955; x-ray at this time was negative. From May to July, 1955, 7 gastric cultures and 9 sputum cultures were negative. X-ray films in October, 1955, indicated probable reactivation in the left upper lung. A sputum culture in November revealed chromogenic mycobacteria and another in January, 1956, exhibited tubercle bacilli. Thoracoplasty involving the fifth rib was performed in May, 1956. X-ray films after surgery failed to indicate reactivation. Treatment by isoniazid and streptomycin was started in June, 1955, and continued until the patient died in October, 1956.

Patient H. P. The diagnosis made by x-ray was bilateral, moderately advanced tuberculosis of lungs active from October, 1949, to January, 1955. Tubercle bacilli were grown on a sputum cultured in November, 1953. X-ray films in June, 1955 showed evidence of inactive tuberculosis. Treatment by *p*-aminosalicylate and streptomycin was started March, 1954, and discontinued January, 1956. Sputum cultured in July, 1955 (strain used in this study), and May, 1956, revealed chromogenic mycobacteria. Three cultures between April and August, 1956, were negative, and x-ray films in August and September showed no evidence of active disease.

Patient F. E. Tuberculosis of lungs was diagnosed by x-ray in May, 1955. One of 3 gastric cultures done at that time revealed chromogenic mycobacteria (strain used in this study); the others were negative. The patient was sent to a sanatorium where 3 additional sputum cultures and a skin test to 0.005 mg. P.P.D. were negative.

Patient H. R. A diagnosis of inactive pulmonary tuberculosis was made by x-ray films taken between June and November, 1955. The chromogenic mycobacteria used in this study were isolated from gastric culture in June, 1955. Four sputa and 7 gastric fluids cultured between July, 1955, and September, 1956, were negative. Treatment with streptomycin and isoniazid was started November, 1953, and discontinued June, 1956. The patient was discharged September, 1956.

Patient J. S. The patient was admitted to the tuberculosis observation unit because sputum culture in June, 1955, revealed chromogenic mycobacteria (strain used in this study), and x-ray films showed a dense area under the anterior fourth rib. One gastric fluid cultured in October, 1955, exhibited chromogenic mycobacteria, and 2 others were negative. No evidence of tuberculosis was seen on x-rays taken between June, 1955, and March, 1956. The patient was discharged in March, 1956. No antimicrobial therapy was given. In June, 1956, x-ray films showed a lesion in the upper left lung with interlobar effusion on the right side. Two gastric fluids cultured in July were negative, but 1 revealed tubercle bacilli; 2 sputa cultured in August and September were negative. In July, a skin test to 0.00002 mg. P.P.D. was positive. Chest x-ray in October, 1956, showed no change. Streptomycin and isoniazid therapy was started July, 1956.

Patient H. C. Chest x-ray on June, 1955, was normal. The patient had a positive skin test to 1:10,000 O.T. Chromogenic mycobacteria were found on sputum culture of June, 1955. The patient was discharged the following month.

Patient G. S. Chest x-ray in November, 1953, showed bronchopneumonia undergoing resolution with right hilar region showing hazing. Six sputum cultures between June, 1954, and October, 1956, were negative. Chromogenic mycobacteria were isolated from a gastric culture in June, 1955 (strain used in this study), and again in October. Seven x-rays taken between December, 1953, and August, 1956, were negative. No antimicrobial therapy was administered.

The control group consisted of 2 photochromogens, P^1 and P^{8*}; 1 *Mycobacterium tuberculosis* (bovine), American Type Collection, No. 9834; 2 *Myco. tuberculosis* (human), a wild

*Obtained from Dr. Ernest H. Runyon, Veterans Administration Hospital, Sunmount, N. Y.

strain (Wayne County General Hospital) and RB199; 1 BCG; and 2 saprophytes, *Mycobacterium phlei* and *Mycobacterium smegmatis*. The latter organisms were obtained from the Department of Bacteriology, University of Michigan Medical School.

The organisms were first grown on Dubos' albumin-liquid medium* containing 0.01 per cent Tween 80 and 5 per cent glycerol. Ten to 14 day cultures were checked for purity and then used for the various tests. Inocula were standardized by the wet-weight method used by the Tuberculosis Laboratory, Communicable Disease Center, United States Public Health Service,[23] except when indicated otherwise.

Culture Media.—Thioglycollate broth (0.75 per cent agar and no dye), Sabouraud's dextrose agar (pH 7.0), Löffler's serum medium,* and trypticase soy broth† were inoculated with 0.1 ml. of 10 to 14 day cultures grown in Dubos' albumin-liquid medium containing 0.01 per cent Tween 80 and 5 per cent glycerol. All cultures were incubated at 37° C. Two sets of Sasano and Medlar's egg medium[24] were seeded similarly, one set was incubated at 25° C. and the other at 37° C.‡ All cultures were observed over a period of 6 weeks.

Cord formation on Dubos' albumin-liquid medium containing 0.01 per cent Tween 80 and 5 per cent glycerol was observed by gross and microscopic examinations of 1, 2, and 3 weeks' growth.

Acid-Alcohol Resistance.—Smears of 1-, 2-, and 3-week cultures grown in Dubos' albumin-liquid medium were stained by the Ziehl-Neelsen technique. Samples were decolorized (3 per cent HCl in 95 per cent alcohol) for periods of 0.5, 2, 4, and 6 hours.

In Vitro Virulence Tests.—The neutral red cytochemical reaction described by Dubos and Middlebrook[25] and the oxidation-reduction test employing redox dyes described by Wilson and associates[26] were done on 2- and 3-week growths of the original isolates and on some organisms recovered after animal passage. The dyes in the latter test were: 3'-chloroindophenol, sodium salt; 2, 6-dibromoindophenol, sodium salt; and indophenol, sodium salt.

Sensitivity to Antimicrobial Agents.—Streptomycin sulfate, sodium *p*-aminosalicylate, and viomycin sulfate were used in final concentrations of 100, 10, 1, and 0.1 μg/ml. Isoniazid was used in final concentrations of 50, 5, 0.5, and 0.05 μg/ml. The combinations tested were *p*-aminosalicylate and streptomycin; *p*-aminosalicylate and isoniazid; *p*-aminosalicylate, streptomycin, and isoniazid. Each combination contained final concentrations of 100, 10, 1, and 0.1 μg/ml. of each drug. Organisms were used in a final concentration of 0.1 mg./ml.; Dubos' albumin-liquid medium containing 0.01 per cent Tween 80 and 5 per cent glycerol served as the test medium. Tubes were observed weekly, and final readings were made after 4 weeks' incubation at 37° C. They were compared visually with controls made to contain 0.1 mg./ml. and 1 mg./ml. of organisms. Growth was verified by microscopic examinations.

Bacteriologic and Histopathologic Examinations.—Groups of 3 tuberculin-negative guinea pigs were injected intraperitoneally with either 0.1, 1, 10, or 20 mg. of organisms making a total of 12 animals for each strain except R. P. The latter was injected in 20 mg. doses in each of 3 animals. The controls consisted of groups of 3 animals each inoculated with either 0.1, 1, or 10 mg. of culture RB199 and of the wild strain of *Myco. tuberculosis* making a total of 9 animals for each strain. Animals from each group were sacrificed at intervals of 1, 2, and 3 months. Infected tissues were homogenized and cultured on 2 to 4 slants of egg medium which were incubated at 37° C. and observed over a period of 3 months.

The tissues of 2 animals, both inoculated with either 1, 10, or 20 mg. of organisms, one infected for 1 month and the other for 3 months, were examined histologically by the Department of Pathology, University of Michigan Medical School. None of the animals injected with 0.1 mg. were submitted for examination. All animals inoculated with strain R. P. and the control animals infected with the wild strain of *Myco. tuberculosis* were sectioned. The photochromogens were not tested in animals.

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*Difco Laboratories, Detroit, Mich.

†Baltimore Biological Laboratories, Baltimore, Md.

‡Typical mammalian tubercle bacilli form smooth, domelike, buff-colored colonies, 2 to 5 mm. in diameter on this medium.

RESULTS

Morphology, Staining, and Cultural Characteristics.—The size and shape of the chromogenic mycobacteria were within the limits of tubercle bacilli except photochromogen P^8 which is a longer and wider rod. Pleomorphism was evident when chromogens were grown on suboptimal media.

Acid-alcohol decolorization was resisted for 6 hours by all the organisms except *Myco. phlei* and *Myco. smegmatis.* The latter failed to resist decolorization beyond 0.5 hour exposure to acid-alcohol.

Growth rate of chromogens at 37° C. and on optimal media was comparable to that of *Myco. tuberculosis;* eugonic and dysgonic types of growth were seen. Chromogens usually required from 1 to 3 days longer to initiate growth at 25° C. than at 37° C. The various control strains of *Myco. tuberculosis* failed to grow at 25° C. but the 2 saprophytes grew quickly and luxuriantly. The scotochromogens formed a deep-yellow to orange pigment on egg medium which intensified at room temperature and when exposed to light, whereas the photochromogens produced buff-colored colonies which turned yellow when exposed to light for 1 or more hours during active growth. Strain P^1 formed smooth, domelike colonies, whereas P^8 produced heaped, rough-edged colonies. The scotochromogens exhibited smooth, slightly raised or domelike colonies except strain H. R., which formed heaped, rough ones. The majority of chromogens emulsified readily in water. The ability to form pigment was retained by the scotochromogens when grown on suboptimal solid media; however, this characteristic was less distinct in liquid suboptimal media.

The growth characteristics of the pigmented mycobacteria and the control organisms are given in Table I. All the scotochromogens grew on 2 or more of the 4 suboptimal media used. However, no one medium could be considered selective for them. Their growth on suboptimal media was initiated later than when identical inocula were made on egg medium or Dubos' albumin-liquid broth, and growth usually increased slightly by the end of the sixth week of cultivation. The 2 saprophytes grew luxuriantly on all media, and they produced pellicles in broth. The 2 photochromogens differed from the scotochromogens in that they failed to grow in thioglycollate broth. In no instance was branching noted by any of the organisms grown on Sabouraud's dextrose agar. The human strains of *Myco. tuberculosis* did not grow on any of the suboptimal media, whereas the bovine strain grew on all but Löffler's serum medium, and BCG grew on all the media to the same extent as did the scotochromogens.

The formation of cords on suitable media is generally attributed to virulent mycobacteria by Middlebrook and associates[27] and Dubos.[28] Only strains H. C. and J. S. formed this pattern after 3 weeks' growth in Dubos' albumin-liquid broth. The other chromogens and the saprophytes exhibited diffuse, granular, or floccular growth patterns, whereas the control strains of *Myco. tuberculosis* produced cords with varying tenacity.

In Vitro Virulence Tests.—Strains H. P., H. R., and J. S. gave positive neutral red tests with original isolates and again when these strains were recovered

from infected animals. The various strains of *Myco. tuberculosis* and the photochromogens also gave positive results indicating virulence, whereas the saprophytes and the other chromogens produced negative reactions.

TABLE I. GROWTH ON SUBOPTIMAL MEDIA

ORGANISM	LÖFFLER'S SERUM MEDIUM	TRYPTICASE SOY BROTH	SABOURAUD'S DEXTROSE AGAR	THIOGLYCOLLATE BROTH
R. P.	+11	+24	-42	-42
H. P.	-42	-42	-42	-42
F. E.	-42	+17	+16	+20
H. R.	+ 8	+17	+5	+20
J. S.	+ 4	+18	+5	+17
H. C.	+ 3	+20	+3	+17
G. S.	-42	+16	+18	+18
P1	+10	+4	+28	-42
P8	+ 7	+7	+24	-42
Myco. tuberculosis var. *bovis*	-42	+17	+5	+20
BCG	+16	+16	+9	+20
Myco. tuberculosis wild strain	-42	-42	-42	-42
Myco. tuberculosis RB199	-42	-42	-42	-42
Myco. phlei	+++ 3	+3	+3	+1
Myco. smegmatis	++ 4	+3	+3	+1

Cultures were incubated at 37° C. and examined at 1- to 3-day intervals until the experiment was terminated on the forty-second day.

Extent of growth is indicated by plus sign (+ to ++++); no growth is indicated by minus sign (-).

Numbers represent the first day growth was evident and verified by microscopic examinations.

The ability to reduce certain redox dyes is a characteristic of saprophytes and avirulent mycobacteria. In this study, strain H. R. failed to decolorize the dyes and strain R. P. partially decolorized them. The other chromogens and saprophytes reduced the dyes, whereas the virulent and attenuated mycobacteria failed to do so.

In Vitro Sensitivity Tests.—The results of the sensitivity tests are given in Tables II and III. Chromogens consistently resisted *p*-aminosalicylate. This correlates with findings reported by Runyon[21] and Wood and associates[4] but not as close to the results reported by Tarshis and Frisch.[18] The use of different media in these tests may explain this discrepancy. Resistance to streptomycin, isoniazid, and viomycin was less consistent and was exhibited in decreasing degree in the order mentioned. Multiple drugs caused the greatest degree of inhibition especially when the combination of *p*-aminosalicylate, streptomycin, and isoniazid was used. All strains, except H. P. and H. R., were recovered before therapy.

TABLE II. IN VITRO RESPONSE TO ANTIMICROBIAL AGENTS

	STREPTOMYCIN				*p*-AMINOSALICYLATE				VIOMYCIN				ISONIAZID				CONTROLS	
	CONC. DRUG = μG/ML.				CONC. DRUG = μG/ML.				CONC. DRUG = μG/ML.				CONC. DRUG = μG/ML.				CONC. ORG. = MG./ML.	
ORGANISM	100	10	1.0	0.1	100	10	1.0	0.1	100	10	1.0	0.1	50	5.0	0.5	0.05	0.1	1.0
R. P.	-	-	+	+	-	+	+	+	-	-	+	+	-	-	+	+	+	+
H. P.	-	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+	+	+
F. E.	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
H. R.	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
J. S.	-	+	+	+	+	+	+	+	-	+	+	+	-	-	-	+	+	+
H. C.	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+
G. S.	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+	+	+
P1	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+
P8	-	-	-	+	+	+	+	+	-	-	-	+	-	-	+	+	+	+
*Myco. tuberculosis** wild strain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Myco. phlei	-	-	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+

Growth is indicated by plus sign (+) and is recorded after 4 weeks' incubation at 37° C.; no growth is indicated by minus sign (-).

*Test was done twice with the same results.

TABLE III. IN VITRO RESPONSE TO COMBINED ANTIMICROBIAL AGENTS

	STREPTOMYCIN AND *p*-AMINOSALICYLATE				*p*-AMINOSALICYLATE AND ISONIAZID				*p*-AMINOSALICYLATE, STREPTOMYCIN AND ISONIAZID				CONTROLS	
	CONC. DRUG = μG/ML.				CONC. DRUG = μG/ML.				CONC. DRUG = μG/ML.				CONC. ORG. = MG./ML.	
ORGANISM	100	10	1.0	0.1	100	10	1.0	0.1	100	10	1.0	0.1	0.1	1.0
R. P.	-	+	+	+	-	-	+	+	-	-	+	+	+	+
H. P.	-	-	+	+	-	-	+	+	-	-	+	+	+	+
F. E.	-	+	+	+	-	-	+	+	-	-	+	+	+	+
H. R.	-	+	+	+	-	+	+	+	-	-	+	+	+	+
J. S.	-	-	+	+	-	-	+	+	-	-	-	+	+	+
H. C.	-	+	+	+	-	+	+	+	-	-	-	+	+	+
G. S.	-	-	-	+	-	+	+	+	-	-	+	+	+	+
P1	-	+	+	+	-	+	+	+	-	-	-	+	+	+
P8	-	-	-	+	-	-	-	+	-	-	-	+	+	+
Myco. phlei	-	-	+	+	-	+	+	+	-	-	-	+	+	+

Growth is indicated by plus sign (+) and is recorded after 4 weeks' incubation at 37° C.; no growth is indicated by minus sign (-).

Tests in Guinea Pigs, Bacteriologic Examinations.—Smears of infected tissues were made at autopsy. They nearly always revealed acid-fast bacilli showing typical morphology when they were found in infected animals sacrificed 1 or 2 months after inoculation. However, organisms were short and

pleomorphic in the lesions of animals killed the third month. Identical morphology was noted in sectioned tissues. The cultures of such lesions nearly always were negative. Scotochromogens were more frequently reisolated from lesions of animals sacrificed the first or second month of infection. In these instances, the number of organisms recovered per gram of tissue was less than when identical organs were infected with *Myco. tuberculosis.*

TABLE IV. RESULTS OBTAINED BY INTRAPERITONEAL INOCULATION OF ORGANISMS INTO GUINEA PIGS

	ANIMALS INOCULATED WITH SCOTOCHROMOGENS			ANIMALS INOCULATED WITH *Myco. tuberculosis*		
DOSE	NUMBER	DEATHS/SURVIVALS	INFECTED/NONINFECTED	NUMBER	DEATHS/SURVIVALS	INFECTED/NONINFECTED
0.1 mg.	18	0/18 (0)*	6/18 (33.3)†	6	6/6 (100.0)	6/6 (100)
1.0 mg.	18	0/18 (0)	14/18 (77.7)	6	5/6‡ (83.3)	6/6 (100)
10.0 mg.	18	0/18 (0)	15/18 (83.3)	6	5/6‡ (83.3)	6/6 (100)
20.0 mg.	21	2/21 (9.5)	20/21 (95.4)	0		

*Per cent deaths.
†Per cent infected.
‡One animal injected with 1.0 mg. and one injected with 10 mg. were killed after 3½ months of infection.

Gross Pathology.—The number and per cent of deaths and infections obtained by challenging guinea pigs to various concentrations of organisms is given in Table IV. The scotochromogens produced a suppurative type response in test animals that was more intensive and involved more organs as the concentration of organisms was increased. In general, the lesions consisted of enlarged mesenteric lymph nodes, omental, peritoneal wall, liver, and splenic abscesses. The spleen became infected only when 10 or 20 mg. inocula were used, and even then it became involved less frequently than the other organs mentioned. Both serosal and parenchymal abscesses were present in infected livers and spleens. For the most part, abscesses contained yellow-colored pus, but occasionally a cheesy substance was found. Smears of this material very frequently revealed acid-fast organisms. In a few instances, when animals were challenged with 20 mg. inocula and sacrificed the third month of infection, necrotic areas and/or nodules were seen on the liver and/or omentum. Abdominal adhesions were seen in animals infected with 1 mg. or more of organisms, and they were most prevalent in those animals killed after 1 or 2 months of infection.

One animal injected with 20 mg. of strain H. R. died 2½ weeks after inoculation. Nodules were found on the spleen, liver, and omentum. Abscess formation was not present. The thoracic cavity contained about 15 ml. of fluid and exhibited acid-fast bacilli by direct examination. The mesenteric lymph nodes were markedly enlarged. Organisms were reisolated from the omental lesions. The other 2 animals similarly inoculated were killed, one after 2 and the other after 3 months of infection. Abscesses were present in the peritoneal wall, omentum, and liver; the direct smears of these lesions revealed acid-fast bacilli that failed to grow when cultured. The same type lesions were noted in the 2 groups of animals inoculated with 1 and 10 mg. of organisms; acid-fast bacilli were found in many lesions that were not recovered on culture. No lesions were found in animals inoculated with 0.1 mg. doses.

TABLE V. CORRELATION BETWEEN EXPERIMENTAL DATA AND CLINICAL DIAGNOSIS

CASE	CLINICAL DIAGNOSIS	IN VITRO VIRULENCE		EXPERIMENTAL ANIMALS			
				BACTERIOLOGY		PATHOLOGY	
		N. R.*	REDOX†	DIRECT SMEAR	CULTURE	TUBERCLES	DEATHS/SURVIVALS
R. P.	Inactive tuberculosis March, 1955; reactivated Jan., 1956; died Oct., 1956	-	±	2/3	1/3	1/3	0/3
H. P.	Inactive tuberculosis June, 1955	+	-	3/6	1/6	1/6	0/6
F. E.	Active tuberculosis May, 1955; sent to sanatorium	-	-	3/6	0/6	3/6	0/6
H. R.	Inactive tuberculosis June, 1955	+	+	5/6	1/6	3/6	1/6
J. S.	No tuberculosis June, 1955; active tuberculosis June, 1956	+	-	2/6	2/6	1/6	0/6
H. C.	No tuberculosis June, 1955; discharged July, 1955	-	-	6/6	3/6	4/6	0/6
G. S.	No tuberculosis June, 1955	-	-	0/0	0/6	2/6	0/6
Myco. tuberculosis (wild strain)	Active tuberculosis June, 1955	+	+	0/0	4/4	3/4	4/4

These tabulations represent on'y those experimental animals that were examined histopathologically.

*Neutral red test.

†Oxidation-reduction test.

Minus sign (-) indicates negative results.

Plus-minus sign (±) indicates doubtful results.

Plus sign (+) indicates positive results.

The first diagnosis given represents the clinical status of the patient at the time the orange-pigmented mycobacteria used in this study were isolated. A second diagnosis is given whenever the status of the patient changed during the course of this study.

One of 3 animals inoculated with 20 mg. of strain H. C. died 5 weeks after challenge. Serosal and parenchymal abscesses were present in the spleen and liver, and a bloody exudate was found. The cultures of these lesions were negative. The other 2 animals similarly treated exhibited abdominal adhesions, peritoneal wall, omental, and liver abscesses. On direct examination these lesions revealed acid-fast organisms; they were reisolated upon culture only in the animal infected for a 1-month period. The 2 groups of animals inoculated with 1 and 10 mg. showed the same type gross lesions containing acid-fast bacilli on direct examination. Organisms were grown from the lesions of the animals infected for 1 and 2 months and not from those infected for 3 months. Two of the 3 animals inoculated with 0.1 mg. doses showed nodules on the omentum.

All but 2 of the control animals died within 3 to 9 weeks after inoculation. One infected with 1 mg. and another with 10 mg. of *Myco. tuberculosis*

were killed 3½ months later. Gross lesions were seen compatible with tuberculosis, and organisms were recovered on culture.

Microscopic Pathology.—The pathologists described the lesions as large chronic abscesses containing purulent exudate within granulomatous walls. The granulation tissue of the abscess wall was not considered entirely characteristic of the granulation tissue found in tuberculosis. The tissue was relatively avascular, but it showed round or oval cells with definite boundaries, unlike epithelioid cells. No Langhans' giant cells and no caseation necrosis were found. A few avascular nodules of epithelioid cells were seen in a few animals, particularly in the liver. These lesions contained no acid-fast organisms, whereas large numbers of organisms were found in the abscess walls. Often animals infected for the longest period revealed organisms that appeared to be disintegrating. No fibrosis or scarring was found. Three of the 4 control animals sectioned showed histologic evidence of tuberculosis.

Correlation of Clinical and Experimental Data.—The experimental data and clinical diagnosis are summarized in Table V. One animal killed by 20 mg. of strain H. C. was not included in the tabulations; it was frozen after death making histologic study impossible. Only experimental animals submitted for histopathologic examination are recorded. These consist of 2 animals infected with 1, 10, or 20 mg. inocula for all strains except R. P. The latter was inoculated in 20 mg. doses into each of 3 animals; all of these were sectioned. Histologic evidence of tuberculosis is based on the presence of epithelioid tubercles and/or presence of acid-fast bacilli in sectioned tissues and by the growth of organisms from these tissues. On this basis, there is no close correlation of tuberculosis between experimental animals and the clinical status of the patients.

DISCUSSION

Several laboratory procedures have been performed on 7 strains of freshly isolated scotochromogens with the aim of possibly using some as laboratory aids for their identification and differentiation.

Scotochromogens have morphologic and staining characteristics similar to tubercle bacilli. Their capacity to resist acid-alcohol decolorization longer than the common saprophytic mycobacteria is useful criteria for their differentiation.

Scotochromogens grow best on optimal media; their growth rate is similar to tubercle bacilli and less like the common saprophytic mycobacteria. Although they are less exacting in nutrient requirements, this does not quite approach the minimal needs of saprophytic mycobacteria. None of the suboptimal media used in this study proved to be selective; however, their use can give helpful information. Temperature requirement is also less critical than is necessary for the growth of tubercle bacilli. This has been found true of other pigmented mycobacteria considered pathogenic for man.[1-4] It is advisable to seed triplicate cultures on an optimal medium of choice, incubating one set at 25° C., one between 30° and 33° C., and another at 37° C. exposing at least the latter set to light after growth has started. This would differentiate

scotochromogens from tubercle bacilli, photochromogens, *Mycobacterium ulcerans*,[1] and *Mycobacterium balnei*.[2] The latter 2 organisms have been found to grow best at 30° to 33° C. and not at 37° C.

The pattern of growth in Dubos' albumin-liquid medium and reactions to in vitro virulence tests are not useful criteria for the differentiation or identification of scotochromogens since results obtained were variable.

Tolerance to antimicrobial agents, especially to *p*-aminosalicylate and less so to streptomycin, is a reasonably consistent finding that helps characterize scotochromogens.

The organisms investigated failed to produce progressive tuberculosis in guinea pigs. Instead a chronic suppurative inflammatory response was produced. The lesions exhibited epithelioid tubercles and atypical giant cells with no evidence of caseation necrosis. In these experimental animals the formation of epithelioid tubercles did not appear to be a reflection of the number of organisms injected. There was no histologic evidence of repair; however, the animals with the longest period of infection revealed short, pleomorphic acid-fast bacilli on direct smears and in stained sections that failed to grow when cultured. It appeared that animals sacrificed 3 months after challenge were making an attempt to overcome the invading organisms.

Other pigmented mycobacteria such as *Myco. ulcerans*,[1] *Myco. balnei*,[2] and *Myco. kansasii*,[3, 4] though associated with human lesions, apparently fail to produce progressive tuberculosis in guinea pigs but they can incite infections in rats, mice, or hamsters. Cellular response in these animals is not entirely characteristic of tuberculosis even though infections can be fatal.

Strain H. R. caused the death of 1 animal when 20 mg. inoculum was used. In this animal the gross lesions were indistinguishable from those produced by tubercle bacilli; histologic examination revealed numerous epithelioid tubercles in the liver and spleen, and the latter exhibited many atypical multinucleated giant cells. Other animals inoculated with this strain were sacrificed and abscess formation was prevalent among the infected ones. Occasional epithelioid tubercles were present in the liver of another animal injected with 20 mg. and killed 3 months later and in the 2 animals inoculated with 1 mg., but none was found in those infected with 10 mg. of organisms. This strain exhibited some cultural properties and in vitro virulence tests that are demonstrated by tubercle bacilli.

Strain H. C. killed 1 animal when 20 mg. of organisms was used; this animal was not sectioned. Two animals similarly inoculated were killed 1 and 3 months later. An occasional epithelioid tubercle was present in the liver of these animals as well as in those that received 1 mg. doses; however, none was found in animals infected with 10 mg. of organisms. All the infected animals showed gross evidence of an inflammatory response. This strain formed loose cords after 3 weeks' growth in Dubos' albumin-liquid medium. Other properties did not conform with those typifying tubercle bacilli.

Orange-pigmented mycobacteria were reisolated subsequently to the ones used in this study in cases R. P., H. P., J. S., and G. S. Case R. P. showed

reactivation of tuberculosis about 2 months after the last pigmented organism was isolated. It is evident that patients harboring scotochromogens may have active tuberculosis concomitantly; they may develop the disease, or they may be in an inactive phase of tuberculosis. Such individuals should be observed closely before considering them free of disease and noninfectious. Furthermore, scotochromogens may have as low a degree of virulence for man as they seem to have for the guinea pig.

The results of experimental studies do not correlate closely with the clinical status of the patients studied; however, an insufficient number of cases have been reviewed to make this an entirely valid conclusion. Continued study of pigmented mycobacteria isolated from man and expanding bacteriologic and animal studies will undoubtedly yield valuable information that may lead to proper and final classification of these organisms.

SUMMARY

On the basis of staining, cultural properties, and in vitro sensitivity reactions to certain antimicrobial agents the scotochromogens are unlike the common saprophytic mycobacteria and tubercle bacilli. They form a group distinct from these. Their bacteriologic characteristics are more like those of photochromogens. A notable difference is that photochromogens need light for synthesis of pigment.

Resistance to acid-alcohol decolorization, tolerance to *p*-aminosalicylate and less so to streptomycin, ability to grow on suboptimal media and at room temperatures, and the capacity to form pigment independent of light are useful criteria for identification of scotochromogens.

A chronic suppurative inflammatory response was produced in guinea pigs. The histologic picture is not typical of tuberculosis although epithelioid tubercles and/or atypical multinucleated giant cells were present in a number of infected animals occurring particularly in the liver.

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THE USE OF BROMSULPHALEIN (B.S.P.) AS AN INDICATOR FOR THE DETERMINATION OF CARDIAC OUTPUT BY THE DYE DILUTION TECHNIQUE

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INTRODUCTION

ALTHOUGH the single injection indicator dilution technique for cardiac output determination has been widely used during the past decade, it suffers from the major limitation that rapid, serial measurements of flow are not feasible with the usual indicators employed. These substances are either cleared from the blood stream too slowly or are potentially noxious. It occurred to us that Bromsulphalein (B.S.P.) might be a more suitable indicator than those commonly used. This innocuous dye, though protein bound, is cleared very rapidly from the blood stream. During the past year this dye has been successfully used in this laboratory as a dilution indicator. In addition, a recent report appeared in the Scandinavian literature with regard to this use of B.S.P.[1] The purpose of this communication is to report our successful application of this dye to the measurement of cardiac output.

MATERIAL AND METHODS

Twelve hospital patients, some with and some without cardiovascular disease, form the basis of this study. Those with cardiovascular disease were not in clinical heart failure. Individual descriptions of the patients are given in Table I.

Cardiac outputs were determined simultaneously in these subjects with Evans blue (T-1824) and B.S.P. Ten milliliters of a normal saline solution containing 4.5 mg. of T-1824 per ml. and 5 mg. of B.S.P. per ml. were injected intravenously from a calibrated syringe in 1 to 2 seconds.

Rapid delivery of the dye into the central venous system was accomplished in each flow determination by injection through a 50 cm. length of (PE 160) polyethylene catheter* whose tip lay at approximately the level of the subclavian vein or the junction of this vessel with the superior vena cava. The catheter was inserted by a modification of the method of Seldinger,[2] with a 75 cm. nylon filament, 0.8 mm. in diameter,† serving as the guide.

Sample Collection.—Serial brachial arterial blood samples were obtained through an 18 gauge, thin-wall Cournand needle, connected with a 2 mm. diameter plastic tubing to a

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*Purchased from the Clay-Adams Company, New York, N. Y.

†Fifty-three pound test monofilament nylon fishing leader.

collecting device similar to that described by Rothe and Sapirstein.[3] The Lucite disk with its heparinized collecting cells was rotated by a constant speed electric motor at the rates of 35, 51, or 70 samples per minute, the more rapid rates being employed where greater cardiac outputs were anticipated.

TABLE I

PATIENT	SEX	AGE	DIAGNOSIS	CARDIAC OUTPUT L./MIN.		DIFFERENCE	
				EVANS BLUE	B.S.P.	C.C.	PER CENT
F. W.	M	51	R.H.D.* with mitral stenosis, emphysema	5.87	6.11	+240	+4.1
W. Mc.	M	43	Bronchogenic carcinoma	10.16	10.43	+270	+2.7
R. S.	F	21	Lymphosarcoma	6.01	5.73	−280	−4.7
R. R.	M	72	A.S.H.D.† with complete A-V dissociation	5.20	5.17	−30	−0.6
O. P.	M	24	R.H.D.* with mitral stenosis	6.51	6.50	−10	−0.2
M. P.	M	71	Hemoptysis of unknown cause	6.44	6.40	−40	−0.6
J. T.	M	46	Diabetes mellitus	5.90	6.09	+190	+3.2
D. K.	F	46	Diabetes mellitus, chronic bronchitis	5.84	5.87	+30	+0.5
J. E.	F	21	Congenital pulmonic stenosis	7.14	7.18	+40	+0.6
H. C.	M	56	Diabetes mellitus	6.60	7.14	+540	+8.2
W. B.	M	47	Duodenal ulcer	4.80	4.86	+60	+1.3
W. D.	M	60	Bronchiectasis, cor pulmonale	6.39	6.62	+230	+3.6
Average				6.41 (±1.34)	6.51 (±1.277)	+103 (±171)	+1.61 (±2.59)

*R.H.D. = rheumatic heart disease.
†A.S.H.D. = arteriosclerotic heart disease.

Dye Analysis.—The need for the separate hematocrit determinations necessary for expression of flows in terms of whole blood was eliminated by the following procedure. Exactly 0.5 ml. of mixed blood, from each sample collected during and immediately following the primary passage of dye, was added to 6 ml. of isotonic saline. Blood cells were removed by centrifugation and dye concentration in the supernatant plasma-saline mixtures was determined with a Beckman DU spectrophotometer. An initial reading to determine the T-1824 concentration in each sample was made at 620 mμ. Following this the optical density of each sample was determined at 565 mμ. A second reading at 565 mμ was made following alkalinization with one drop of saturated sodium hydroxide solution to develop the B.S.P. color. The difference between the first and second readings at 565 mμ was assumed to represent the optical density due to B.S.P. Blanks were prepared from blood samples collected just prior to appearance of dye and were treated in exactly the same manner. In preparation of the standard the same quantity of dye as had been injected into the subject was introduced through the previously used syringe and catheter into a 1,000 ml. volumetric flask and made up to volume with isotonic saline. A 0.5 ml. aliquot of this solution was added to 5.5 ml. of isotonic saline and 0.5 ml. of whole blood also collected prior to the appearance of dye. This sample was then handled the same as the blank and the other samples.

Calculation of Outputs.—The optical density of the blank at 620 mμ was subtracted from all other readings at this wave length. The same was done at 565 mμ before and after alkalinization. The resulting net concentrations of Evans blue and B.S.P., expressed in optical density units, were then individually plotted logarithmically against time, and a line was drawn connecting all points. The slope of the descending limb of each dye curve, established by 4 or more points, was extrapolated beyond the point of gross recirculation. Flow was calculated by essentially the same formula as that used by Rothe and Sapirstein,[3] which is as follows: $Q = \frac{I \times V \times n}{A + B}$ where Q = cardiac output in liters/minute; I = optical density of the standard sample; V = dilution factor of the standard sample (in

these studies 1.0 liter); n = sampling rate as number of samples per minute; A = sum of the optical densities of samples collected prior to recirculation; B = sum of the optical densities of extrapolated points, read from the curve, using the same time intervals as in A.

Serial Output Determination.—In one patient without cardiovascular disease 5 consecutive flow determinations were made within 12 minutes. Using the previously described injection technique, 1 ml. of isotonic saline containing 50 mg. of B.S.P. was injected in each of the 5 measurements. The collected samples, the blank, and the standard were treated as described above except that only a single spectrophotometric reading was necessary. This was made at 565 mμ following alkalinization. The same method as described above was used for output calculation except that only net concentrations at 565 mμ after alkalinization were used.

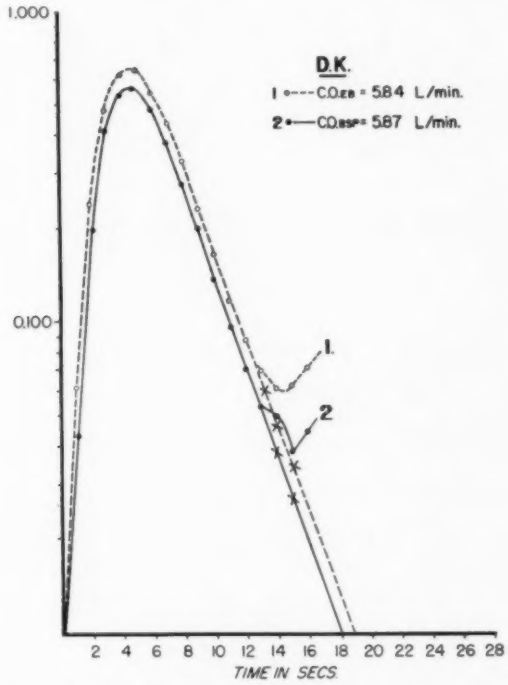

Fig. 1.—Simultaneous Evans blue and B.S.P. dye dilution curves in a patient without cardiovascular disease. The ordinate, a logarithmic scale, represents dye concentration in optical density units. The abscissa represents time in seconds. Points denoted by X in each curve are on the extrapolated portions.

B.S.P. Disappearance.—The rate of B.S.P. disappearance was determined in one normal subject, in one patient with severe hepatic cirrhosis, and in another with congestive heart failure. Following the injection of 50 mg. of B.S.P. by the same technique as used in the output determinations, 5 ml. arterial blood samples were collected at 30-second intervals beginning at 1 and continuing to 10 minutes after injection. All samples were centrifuged and, following alkalinization of the undiluted plasma, B.S.P. concentration was determined at 565 mμ. The optical density of plasma obtained prior to injection served as a blank and was subtracted from each reading. These net B.S.P. concentrations were then plotted logarithmically against time.

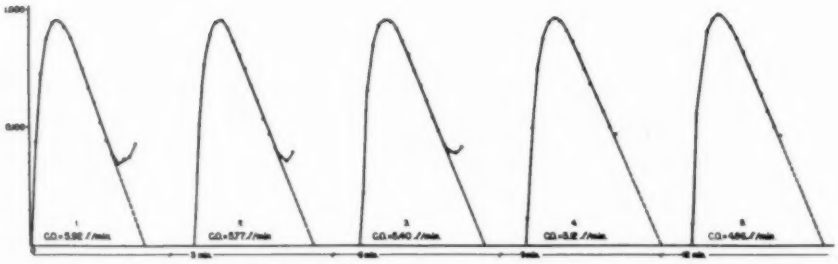

Fig. 2.—Five consecutive B.S.P. dilution curves obtained in a single normal subject in 12 minutes. The ordinate is the same as Fig. 1. The abscissa represents an extended time base in minutes. Each curve, however, has a time base of approximately 20 seconds as in Fig. 1. The extrapolated portion of each curve is represented by the broken line.

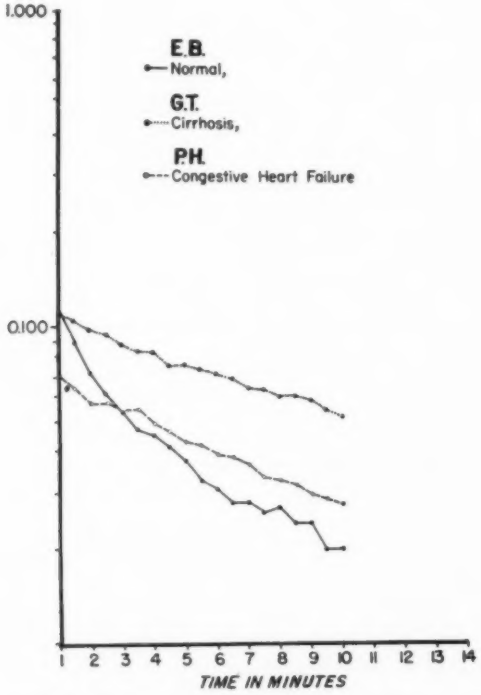

Fig. 3.—Plasma B.S.P. disappearance curves in 3 subjects as indicated. The ordinate, a logarithmic scale, represents dye concentration in optical density units. The abscissa represents time in minutes following dye injection.

RESULTS

The results of the simultaneous B.S.P. and Evans blue flow determinations are summarized in Table I. It can be seen that there is close agreement between the two outputs both in patients with and without cardiovascular disease. Not only is there no systematic difference between the two but also the actual differences noted are within the accepted error of the dye method.

The similarity of the time relationships of the simultaneous B.S.P. and Evans blue curves can be seen in Fig. 1. This agreement was noted in all determinations.

Five successive B.S.P. outputs done in a single patient within 12 minutes are illustrated in Fig. 2. It will be noted that the background due to circulating dye was 0.130 optical density units at the beginning of the fifth determination, compared to 0.051 optical density units at the beginning of the second determination.

Fig. 3 shows B.S.P. disappearance curves in the normal subject, the patient with congestive heart failure, and in the patient with far-advanced Laennec's cirrhosis. Extrapolation of the curve obtained in the normal individual over the first 4 minutes following injection results in a plasma disappearance rate of approximately 20 per cent per minute. The rate in the congestive heart failure patient during this same interval is 9 per cent while that in the patient with cirrhosis is 8 per cent.

DISCUSSION

Since Moore, Kinsman, Hamilton, and Spurling's[4] report on the use of the dilution method for measurement of cardiac output in man, probably the most widely used indicator has been Evans blue (T-1824). Its chief disadvantage, however, is that the body apparently has no efficient mechanism for its removal and therefore a portion of the dye may remain within the blood stream and interstitial space for several weeks following a single injection. This limits one's ability to make rapid serial determinations without producing bluish discoloration of the skin. In attempts to avoid this objection, other substances more rapidly cleared have been employed including other dyes, radioisotopes, and saline solutions.[5] The difficulties encountered in the use of these other compounds, however, have been the inaccuracy of the measurement of their concentrations in blood and/or their potentially harmful effects. One possible exception to this is indigo carmine as reported by Lacy, Ugaz, and Newman.[6] These workers found good agreement between dilution curve areas simultaneously determined with this dye and iodinated (I^{131}) human serum albumin.

We have found that Bromsulphalein has the same distribution in blood in vitro as Evans blue. The identical time courses of the plotted output curves as well as the excellent agreement of calculated flow values obtained when Evans blue and B.S.P. were injected simultaneously seem to indicate that B.S.P. behaves similarly to Evans blue during passage through the heart and lungs.

The advantages of B.S.P., however, are that it is not retained within the vascular system for prolonged periods of time and that it can be accurately

measured spectrophotometrically. Ingelfinger, Bradley, and associates[7] have shown that this dye is rapidly removed from the blood stream by the liver and other organs. They noted that following intravenous injections of 150 mg. per M.^{2} of body surface area in normal subjects, this dye disappears from the plasma at an average rate of 13.6 per cent per minute. Although we find it difficult to make a reliable extrapolation to the time of instantaneous mixing, our data in Fig. 3 indicate that the disappearance rate, at least during the first 4 minutes, is slightly greater following injections of 50 mg. in one normal subject. It should be pointed out however that these workers used a later portion of the plasma disappearance curve for their extrapolations. This rapid clearance of B.S.P. permits repeated determinations of blood flow in a single patient within a short period of time without the problems associated with progressive accumulation of indicator in the blood stream. Illustrating this point the 5 determinations of cardiac output, shown in Fig. 2, were made within 12 minutes. Even after 4 flow determinations in 9 minutes the circulating blood background of dye was only 13 per cent of the peak of the fifth dilution curve. This is not a large blood background and can be easily and accurately corrected for. The output progressively decreased to the extent of one liter during the entire period. The most likely explanation for this decrease would appear to be the blood loss, estimated at about 300 ml., which occurred during the study. This mechanism has also been suggested by Lawson and his associates[8] to explain such a finding. It should be pointed out that no attempt was made to minimize blood loss during this observation and undoubtedly the loss can be reduced as experience is gained with the technique.

Since B.S.P. is removed from the blood stream primarily by the liver, one might question its use for repeated cardiac output measurements in patients with liver disease where prolonged dye retention might be expected. We have studied the rate of B.S.P. clearance in one patient with advanced hepatic disease. The disappearance curve, shown in Fig. 3, indicates that the clearance rate in this individual is about one-half that observed in the normal subject but the concentration of dye is changing so slowly that a stable blood background would be present one minute after injection. This could be easily corrected for the same as in the normal subject and thus rapid serial flow determinations would be possible. In congestive heart failure the rate of B.S.P. excretion is decreased though not to the extent observed in liver disease. The background is likewise stable in this condition and thus repeated flow determinations are theoretically possible.

B.S.P. has the same limitations in the determination of cardiac output in most patients with congestive heart failure as Evans blue or other dilution indicators. Probably because of the increased volume of blood in the heart and lungs in this condition, dilution curves have characteristically extended time bases and accurate detection of gross recirculation on the descending slopes is difficult or impossible. Undetected recirculation will enlarge the area under the curve and the output will be erroneously low in such a situation.[5]

Our results indicate that B.S.P. can be employed as a dilution indicator in any situation where Evans blue is satisfactory.

SUMMARY

1. In a series of 12 patients, some with and some without cardiovascular disease, cardiac output as measured by Bromsulphalein (B.S.P.) injection has been found to agree well with that simultaneously determined with Evans blue (T-1824).

2. The advantage of B.S.P. as a dilution indicator for rapid, repeated flow measurements has been shown by the fact that 5 cardiac output determinations were made in a single patient within 12 minutes.

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LABORATORY METHODS

THE PHOTOMETRIC MICRODETERMINATION OF BLOOD GLUCOSE WITH GLUCOSE OXIDASE

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THE determination of "glucose" in blood, and in other biologic fluids, is undoubtedly the most frequently performed procedure in the clinical laboratory. It is, therefore, unfortunate that for the sake of expediency such analyses are being performed with procedures which are relatively *nonspecific* for glucose.[1] The most widely used and recommended methods for "glucose" or "reducing sugars" are those based either on ferricyanide[2-4] or cupric ion[5-7] reduction in alkaline medium. Such oxidation-reduction systems are not only capable of oxidizing glucose but many other compounds containing the carbonyl ($>C=O$) grouping.[8, 9]

Attempts to increase the specificity of the determination of "glucose" in blood have been directed mainly toward the preparation of filtrates which remove *some* of these reducing substances *prior* to the analysis rather than toward more specific reagents.[10, 11] This development led to the widespread adoption of the Nelson-Somogyi method[12] by many laboratories as a routine procedure since the lower copper reduction values obtained, as compared with the original Folin-Wu tungstic acid filtrate procedure,[5] seemed more in keeping with the "true" glucose values obtained by yeast fermentation.[10]

More recently a number of other procedures have been proposed for the determination of "glucose" in biologic fluids based on condensation reactions of the aldehyde group of carbohydrates with phenols in strong acid solution, e.g., the anthrone reaction[13,14] and the thymol-sulfuric acid reagent.[15] Such procedures not only have the disadvantage of reagent instability and of nonspecificity for glucose, but also are conducted in such high acid concentrations as to make them difficult to handle on a routine basis and to so standardize conditions as to obtain reproducible results.

Up to the introduction of specific enzymatic methods for glucose, the only acceptable measure of "true" glucose was that obtained by estimating the apparent glucose content of plasma filtrates by reduction methods before and

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after fermenting the glucose by means of yeast.[16] This method never achieved widespread use in clinical laboratories since the procedure is also nonspecific and is so time-consuming as to make it impractical for routine work.

The determination of "true" glucose as a practical, routine laboratory procedure became a distinct possibility with the discovery of a specific enzyme for glucose, i.e., glucose oxidase (notatin), by Müller[17] and its preparation in a highly purified form by Coulthard and associates.[18] The pronounced specificity of the enzyme for beta glucose was established by the studies of Keilin and Hartree[19] on about 50 sugars and their derivatives, as well as for all 8 D-aldohexoses.[20] These authors[21] also used the enzyme for the determination of glucose in biologic material with a manometric technique and for the study of the kinetics of different enzyme reactions which involve the liberation of glucose from polysaccharides, phosphorylated sugars, and glucosides.

The most significant advance toward a routine "true" glucose procedure, based upon colorimetric analysis, came from the suggestion of Keston[22] for the simultaneous use of 2 enzymes in the following sequence of reactions:

$$\text{glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{chromogenic oxygen acceptor} \xrightarrow{\text{peroxidase}} \text{chromogen [blue, orange, etc.]}$$

[*o*-tolidine
o-dianisidine, etc.]

These reactions have been used by Keston[22] and Comer[23] for the semiquantitative, specific determination of glucose in urine by means of filter paper strips impregnated with the necessary reagents and by White and Secor[24] for the detection of glucose spots on paper chromatograms.

The only published material which has attempted to employ the two enzyme system of Keston[22] as a routine analytical method for the specific determination of glucose in blood and urine is that of Teller.[25] Our investigations have shown that the method cannot be applied directly to serum or plasma due to the presence of substances inhibitory to the enzyme which are removed by protein precipitation. Studies of the kinetics of the reaction at different glucose concentrations and temperatures indicate that the reaction rate at a 10-minute time interval is markedly affected by a change in both these parameters and that reproducible, stoichiometric values are best obtained at longer incubation periods (30 minutes) and higher temperatures (37° C.). The method, finally evolved, is not only specific for glucose but more rapid and precise than the commonly employed Nelson-Somogyi[12] procedure and, therefore, should be of great value for research work in carbohydrate metabolism. With minor variations in the technique, it can also be used as a routine clinical method for large laboratories performing 100 or more glucose determinations daily.

MATERIALS AND METHODS

A. *Reagents and Apparatus.*—1. Plasma, heparinized-siliconated. Fill a series of test tubes to the brim with a 1:10 dilution of siliclad* in distilled water and let stand for about 3 minutes. Repour siliclad solution back into its original container and dry tubes in an oven at 60° C. Add 0.10 ml. of a solution of sodium heparin† containing 1,000 μg/ml., to each tube and again dry in an oven at 60° C. Five to ten milliliters of freshly drawn venous blood, when added to a tube and mixed by gentle inversion, will not clot for periods from 12 to 24 hours. Plasma obtained by centrifuging such tubes at 2,000 r.p.m. for 5 minutes may be utilized for practically all biochemical determinations except for the total protein and protein flocculation reactions. Oxalated plasma and heparinized whole blood were also employed in some of the comparison studies.

2. Zinc sulfate, 5 per cent solution of reagent grade $ZnSO_4 \cdot 7H_2O$. This solution was used for the research method employing a spectrophotometer.

3. Barium hydroxide 0.3 N solution; 10.0 ml. of this solution should exactly neutralize a 10.0 ml. aliquot of the 5 per cent zinc sulfate solution using phenolphthalein as an indicator. This solution was employed for the research method using a spectrophotometer.

4. Cadmium sulfate, 0.25 per cent solution of 3 $CdSO_4 \cdot 8\ H_2O$. For use in the routine method using a photoelectric colorimeter.

5. Sodium hydroxide, approximately 0.12 N; 1.0 ml. should just neutralize 7.0 ml. of the cadmium sulfate solution using phenolphthalein as an indicator. This sodium hydroxide solution was used for the routine procedure since it gives a more gelatinous precipitate which yields particle-free filtrates upon decantation. The more alkaline pH of these filtrates and the absence of Ba++ ions eliminates the formation of turbid solutions during the enzymatic reactions.

6. Glucostat reagent.‡ The reagent is freshly prepared in sufficient quantity for each day's run exactly as described in the accompanying circular. It contains glucose oxidase, horseradish peroxidases, and phosphate buffer in one vial and *O*-dianisidine in a second vial. Each set of two vials is sufficient for 100 ml. of reagent. For routine determinations, it is desirable to prepare a more concentrated reagent by diluting the contents of the vials to 47.5 ml. plus 2.5 ml. of 0.4 M phosphate buffer, pH 7.0 (139.2 Gm. Na_2HPO_4 and 20.5 Gm. KH_2PO_4 per liter of solution).

7. Glucose standards: 100 mg. per cent and 200 mg. per cent glucose solutions. These are prepared by dilution with 0.1 per cent benzoic acid from a stock standard of 1.000 Gm. per cent glucose (reagent grade) made up in 0.10 per cent benzoic acid solution and are stable at room temperature.

8. Sulfuric acid: 0.50 N solution.

9. Spectrophotometers: Beckman D.U. or equivalent instrument for use in research studies.

10. Photoelectric colorimeter: Klett-Summerson or equivalent instrument for use in routine analysis.

B. *Methods.*—

1. *Glucose oxidase research method for use with a spectrophotometer:* Prepare the filtrate by adding to each 0.50 ml. of plasma (or serum), 7.50 ml. of distilled water and 1.00 ml. each of the 5 per cent zinc sulfate and barium hydroxide. When whole blood is employed the red cells must be completely laked by vigorous shaking after the water addition. Mix and centrifuge, or filter, the precipitate formed.

To 1.00 ml. of the 1:20 Somogyi zinc filtrate, add 6.0 ml. of dilute glucostat reagent, mix, and let tube stand in a water bath at 37° C. for 30 minutes. Then add 1.0 ml. of 0.5 N sulfuric acid solution and mix by inversion. Let tube stand at room temperature for 5 minutes and read in the Beckman D.U. at 395 mμ against a distilled water blank treated in the same

*Clay-Adams Company, New York, N. Y.
†Merck & Company, Inc., Rahway, N. J.
‡Obtainable from the Worthington Biochemical Corporation, Freehold, N. J.

manner as the plasma sample. Duplicate 100 and 200 mg. per cent glucose standards are also run with the procedure simultaneously with each set of unknowns. Optical density readings are directly proportional to glucose concentrations in the 25 to 400 mg. per cent range.

2. *Glucose oxidase routine method for use with a colorimeter:* To 0.10 ml. of plasma or serum, add 7.0 ml. of the cadmium sulfate solution and 1.0 ml. of the sodium hydroxide solution. Mix and let stand 10 minutes or longer at room temperature. Mix again and centrifuge at 3,000 r.p.m. for 10 minutes to pack down the precipitate. Decant the supernatant fluid into another tube in such a manner as to avoid carrying over particles of the precipitate but without concern as to leaving behind volumes of fluid less than 0.5 ml. Add 2.0 ml. of the *concentrated* glucose oxidase reagent, mix, and incubate at 37° C. for 30 minutes. Add 1.0 ml. of 0.5 N sulfuric acid and mix by inversion. Let tube stand for 5 minutes at room temperature and read in a Klett-Summerson colorimeter with a No. 42 filter against a distilled water blank prepared in a similar manner. Duplicate 100 and 200 mg. per cent glucose standards are also prepared simultaneously with each set of unknowns. Glucose concentrations are directly proportional to the colorimetric readings in the 50 to 300 mg. per cent range.

RESULTS

Studies of Factors Which Influence the Colorimetric Procedure. —

1. *Spectral curves:* Optical density readings were made with the Beckman D.U. over the range from 350 mμ to 600 mμ for the yellow color obtained with the glucose oxidase method using glucose concentrations of 50, 100, and 200 mg. per cent. The results obtained show a maximum of approximately 395 mμ for each concentration. Subsequent readings, therefore, were all measured at this wave length.

2. *Variation of time of incubation:* The glucose oxidase procedure, described previously in B, 1, was run for a series of glucose standards containing 50, 100, and 200 mg. per cent, respectively, except that the incubation time at 28° C. was varied from 1½ to 60 minutes before acid addition. The results obtained in these studies show that the rate of increase of color development decreases and approaches a constant value after a 30-minute incubation period which is independent of the glucose concentration employed. Stoichiometric values at these concentrations were obtained only *after* a 20- to 60-minute incubation period. The decrease in the rate of color development permits the stopping of the enzymatic reaction by acid addition over a 5-minute time interval without appreciable loss in accuracy of the procedure.

3. *Variation of acid concentration:* The addition of acid is necessary to stabilize the colorimetric readings and serves both to stop the enzymatic reactions and to eliminate any turbidity formed during the reaction. The effect of acid addition, i.e., sulfuric acid, was studied over the range from 0 to 1.1 N of final acid concentration in an 8.00 ml. volume. The results obtained show that the optical density readings reach a maximum plateau between 0.04 to 0.15 N and then decline steadily with increasing acid concentration. Based on these data, the final acid concentration employed for the glucose determinations corresponds to approximately 0.06 N sulfuric acid.

4. *Variation of reading time after acid addition:* The effect on the optical density readings of time of standing after acid addition was studied over a time interval of 1 hour. The data obtained demonstrate that the optical density values reach a maximum plateau between 5 and 30 minutes after acid addition.

5. *Variation of temperature of incubation:* The effect of variation of time of incubation over a 1 hour period was studied at temperatures of 28°, 37°, and 56° C. From the data obtained in these experiments it was found that the maximal reaction was at 37° over a 30- to 60-minute time interval and that an incubation time of 30 minutes at 37° gave about the same optical density value as did a 60-minute incubation period at 28° C.

6. *Variation of glucose concentration:* The effect of varying concentration over a range of values from 0.0125 mg. to 0.200 mg. glucose, i.e., from 25 to 400 mg. per cent based upon a preparation of a 1:20 Somogyi filtrate, was studied with the procedure described in paragraph B, 1. The data obtained in these runs are plotted in Fig. 1 as optical density readings versus glucose concentration, and shows that the reaction follows Beer's law over this concentration range.

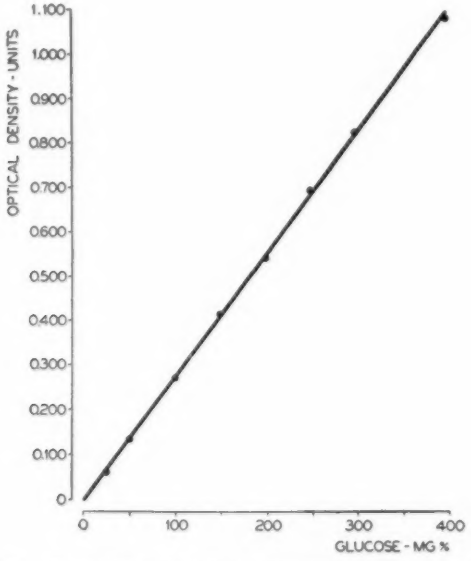

Fig. 1.—Effect of variation of glucose concentration from 0.0125 mg. to 0.200 mg. (or 25 to 400 mg. per cent as based on a 1:20 plasma zinc filtrate) on the optical density readings obtained.

Application of Method to the Determination of the Glucose Content of Plasma.—

1. *Direct plasma determination:* Teller[25] had previously proposed a procedure for the direct colorimetric determination of the glucose content of serum by means of the enzymatic glucose oxidase method in comparison with the Nelson-Somogyi method.[12] Since it would be desirable to avoid the time-consuming protein precipitation step, this problem was reinvestigated by us, using the procedure described under B, 1, on aliquots of 0.050 ml. of plasma in comparison with the values obtained with 1:20 filtrates of the same plasma. A mean value

of 71.9 mg. per cent was obtained with the direct method for the 13 plasmas using serum directly as compared to a mean value of 99.7 mg. per cent when the method was performed with zinc filtrates of these same plasmas. The consistently lower values obtained with plasma filtrates (average = 27.8 mg. per cent), indicated the presence of an enzyme inhibitor so that all subsequent determinations were performed on Somogyi zinc filtrates.

2. *Reproducibility of method for plasma filtrates:* Triplicate glucose determinations were performed with the glucose oxidase method (Materials and Methods, B, 1) on zinc filtrates prepared from 15 separate bottles of a dried serum preparation* all bearing the same lot number. The results obtained in these runs gave a mean value of 93.8 mg. per cent ± 2.1 s.d. with the Nelson-Somogyi method and 79.9 mg. per cent ± 2.3 s.d. with the glucose oxidase method for the same filtrates. There was present in this material 14.0 mg. per cent ± 3.1 s.d. of nonglucose reducing substances. The data obtained show that the standard deviation from the mean is about the same for each procedure and that the average error for a determination, run in triplicate, is about 2 per cent for either method.

TABLE I. RECOVERY OF GLUCOSE ADDED TO PLASMA WITH THE COLORIMETRIC GLUCOSE OXIDASE METHOD

PLASMA SAMPLE NO.	PLASMA GLUCOSE (MG. PER CENT)	GLUCOSE ADDED (MG. PER CENT)	GLUCOSE FOUND (MG. PER CENT)	GLUCOSE CALCULATED (MG. PER CENT)	RECOVERY (PER CENT)
1	124.0	0.0	—	—	—
	124.0	50.0	86.0	87.0	99.0
	124.0	100.0	110.0	112.0	98.4
	124.0	200.0	164.0	162.0	101.0
2	275.0	0.0	—	—	—
	275.0	50.0	166.0	163.0	101.7
	275.0	100.0	188.0	188.0	100.0
3	123.0	0.0	—	—	—
	123.0	50.0	88.0	87.0	101.0
	123.0	100.0	110.0	112.0	98.2
	123.0	200.0	158.0	162.0	97.5
4	194.5	0.0	—	—	—
	194.5	50.0	120.0	122.3	98.2
	194.5	100.0	142.0	147.3	96.6
	194.5	200.0	194.0	197.3	98.6
5	93.4	0.0	—	—	—
	93.4	50.0	69.5	71.7	97.0
	93.4	100.0	96.7	96.7	100.0
	93.4	200.0	143.0	146.7	97.5
6	175.0	0.0	—	—	—
	175.0	50.0	110.0	112.5	97.8
	175.0	100.0	135.0	137.5	98.2
	175.0	200.0	185.0	187.5	98.6

3. *Recovery of glucose added to plasma:* To one volume of plasma was added an equal volume of a standard solution containing either 50, 100, or 200 mg. per cent of glucose. A 1:20 Somogyi zinc filtrate was then prepared and the glucose content of the mixture determined with the glucose oxidase procedure

*Versatol; Warner-Chilcott Laboratories, New York, N. Y.

(B, 1). Table I shows the calculated and experimentally recovered glucose values obtained for 6 different plasma samples with this method. The amounts of glucose recovered range from 97 to 102 per cent and provide experimental evidence against the presence of enzyme inhibitory substances in the filtrates.

4. *Normal plasma glucose values:* Heparinized-siliconated plasma was obtained from 33 normal individuals of both sexes whose ages ranged from 20 to 55 years of age. The zinc filtrates were prepared within 5 to 10 minutes after venous puncture to minimize changes due to glycolysis. The same filtrates were run in triplicate both with the Nelson-Somogyi[12] and the glucose oxidase methods. A statistical analysis of the data obtained in these experiments is given in Table II and some representative individual results are shown in Table III.

TABLE II. STATISTICAL ANALYSIS OF PLASMA GLUCOSE VALUES OBTAINED WITH GLUCOSE OXIDASE AND NELSON-SOMOGYI METHODS FOR NORMAL, NONDIABETIC, AND DIABETIC INDIVIDUALS

TYPE AND NO. OF SAMPLE	(1) NELSON-SOMOGYI METHOD MEAN ± S.D. (MG. PER CENT)	(2) GLUCOSE OXIDASE METHOD MEAN ± S.D. (MG. PER CENT)	DIFFERENCES 1–2 MEAN ± S.D. (MG. PER CENT)
Normal plasma 33	99.5 ± 8.8	90.9 ± 7.9*	+8.6 ± 3.3
Nondiabetic plasmas 36	96.8 ± 9.1	87.1 ± 8.8†	+9.9 ± 2.8
Diabetic plasmas 36	168.4 ± 56.7	147.5 ± 51.9	+20.9 ± 8.8

*Normal range (general population) = mean ± 2 s.d. = 75 to 107 mg. per cent.
†Normal range (hospitalized patients) = mean ± 2 s.d. = 69 to 105 mg. per cent.

5. *Glucose values of nondiabetic patients:* Plasma zinc filtrates of 36 nondiabetic, hospitalized patients were analyzed in triplicate for glucose content with both the glucose oxidase and Nelson-Somogyi procedures. A statistical analysis of the data obtained in these runs is given in Table II and some of the individual data are illustrated in Table III.

6. *Glucose values of diabetic patients:* The zinc filtrates of the plasmas obtained from 36 hospitalized, diabetic patients were analyzed in triplicate for their glucose content with both the Nelson-Somogyi and glucose oxidase methods. A statistical analysis of the data obtained in these experiments is also presented in Table II and some of the individual results are shown in Table III.

7. *Comparison of results obtained with whole blood and plasma:* Heparinized venous blood samples were obtained from 6 nondiabetic and 7 diabetic patients. Zinc filtrates, 1:20, were prepared in triplicate from the whole blood samples making certain that all the cells were laked by shaking thoroughly after the water addition. The blood samples were then centrifuged and zinc filtrates prepared in triplicate from the plasmas obtained. Glucose determinations were then performed with the glucose oxidase method for each filtrate. Mean values obtained for the 6 nondiabetic patients were 76.7 mg. per cent (whole blood) and 79.7 mg. per cent (plasma). For the 7 diabetic patients, the mean values were 180 mg. per cent (whole blood) and 185 mg. per cent (plasma). These

results indicate that whole blood may be used instead of plasma, e.g., finger tip blood for the microglucose tolerance procedure, with the glucose oxidase method, although slightly lower values (3 to 5 mg. per cent) are obtained.

Heparinized and oxalated plasmas were simultaneously obtained from venous blood samples which were drawn from 6 additional patients and their glucose contents determined in the same manner as described above. A mean value of 96.3 mg. per cent was found for the oxalated plasma samples as compared to 101 mg. per cent for the heparinized samples. The substitution of oxalated for heparinized plasma in the glucose oxidase method yields glucose values which are about 5 mg. per cent lower on the average.

TABLE III. INDIVIDUAL GLUCOSE VALUES OF PLASMA ZINC FILTRATES

PLASMA SAMPLE NO.	(1) NELSON-SOMOGYI METHOD (MG. PER CENT)	(2) GLUCOSE OXIDASE METHOD (MG. PER CENT)	DIFFERENCE 1–2 (MG. PER CENT)
	(a) Normal Individuals		
1	106.0	98.6	+7.4
2	85.4	78.8	+6.6
3	103.0	93.0	+10.0
4	91.0	79.8	+11.2
5	84.8	81.8	+3.0
6	97.6	89.0	+8.6
7	106.0	94.2	+12.0
8	116.0	103.0	+13.0
9	91.8	82.6	+9.2
10	111.4	102.0	+9.4
	(b) Nondiabetic Patients		
11	92.2	82.6	+9.6
12	93.5	86.4	+7.1
13	113.0	103.0	+10.0
14	99.5	84.4	+13.1
15	107.0	98.4	+8.6
16	105.0	94.8	+10.2
17	91.5	78.3	+13.2
18	93.0	79.7	+13.3
19	107.0	94.5	+12.5
20	88.6	77.8	+10.8
	(c) Diabetic Patients		
21	242.0	204.0	+38.0
22	266.0	238.0	+28.0
23	277.0	247.0	+30.0
24	315.0	267.0	+48.0
25	206.0	191.0	+15.0
26	198.0	191.0	+7.0
27	166.0	152.0	+14.0
28	100.0	81.5	+18.5
29	126.0	105.0	+21.0
30	113.0	92.7	+20.3

DISCUSSION

The idea of obtaining plasma by collecting venous blood in siliconated tubes containing minute amounts (100 μg) of sodium heparin was suggested to us by Dr. Samuel Losner* of this laboratory as a result of his extensive work in the field of blood clotting.[26] In routine clinical work, use of plasma has the

*Deceased Jan. 14, 1957.

advantage of permitting the analysis to be started within a few minutes after the sample reaches the laboratory. This is especially important in the determination of glucose since an appreciable loss in blood sugar may occur during the interval required for the blood to clot prior to centrifugation where serum is used in the analysis.[1] Heparinized-siliconated plasma may be used for practically all routine biochemical procedures, including sodium and potassium analyses, except for the total protein, or protein flocculation reactions, because of the presence of fibrinogen. The use of plasma, instead of serum or whole blood, for the glucose determinations would serve to account for the higher mean value, i.e., 99.5 mg. per cent, obtained for the 33 fasting normal individuals with the Nelson-Somogyi method (Table II) than the mean value of 90 mg. per cent reported by others.[27] Glycolysis upon standing would also account for the somewhat lower mean results, i.e., 3 to 4 mg. per cent, obtained for the nondiabetic patient's plasma with both the glucose oxidase and Nelson-Somogyi methods because of the time lapse between the drawing of the blood and its reaching the laboratory.[1] It is, therefore, more accurate to use as a normal range the mean glucose value ± 2 standard deviations of the nondiabetic patients, rather than that of the group of normal individuals, in determining whether a hospitalized individual has an elevated blood sugar. The normal range of 69 to 105 mg. per cent was selected on this basis. This is almost identical with the range reported for the Benedict copper reduction method using sodium bisulfite.[1] However, in surveys of the normal healthy population for diabetics, as in public health clinics, it would be advisable to use the normal group in determining the normal range, i.e., 75 to 107 mg. per cent.

Perhaps, the most interesting part of this data is the consistently higher glucose values obtained for all plasmas thus far studied with the Nelson-Somogyi procedure as compared to the glucose oxidase method. For normal plasmas, this difference amounts to about 9 per cent of the total reducing substance in the zinc filtrates, or 8.6 out of 99.5 mg. per cent on the average. For the nondiabetic patients, the amount of reducing substances not attributable to glucose (or saccharoids) is slightly higher, i.e., 9.9 out of 96.8 mg. per cent (or 10.2 per cent) on the average. Whereas, for the diabetic group the differences between the Nelson-Somogyi and glucose oxidase methods amount to an average value of about 21 out of 168 mg. per cent or 12.5 per cent of nonglucose reducing substances. The marked increase of nonglucose reducing substances in diabetics, as compared to normal individuals or nondiabetic patients, is more evident from the data in Table III in which some of the individual results are listed for the 3 groups studied. In no instance did the plasma of a normal individual or nondiabetic patient exhibit a difference value greater than + 16 mg. per cent; whereas diabetic patients have difference values greater than + 16 mg. per cent in about one-half of the cases. As can be seen from the individual diabetic values in Table III, *c*, some cases (25, 26, and 27) with elevated glucose values have difference values less than + 16 mg. per cent while other cases with glucose values in the normal range (28, 29,

and 30) have difference values greater than + 16 mg. per cent. The exact nature of the increased saccharoids[8] in the plasma filtrates of some diabetic patients, but not in others, is presently under investigation.

The analytical results presented in this paper were all performed with procedure B, 1 using the Beckman D.U. at 395 mμ to perform the spectrophotometric readings. For the past few months, procedure B, 2 has been used as the routine procedure for glucose determinations. It makes use of 0.10 ml. heparinized-siliconated plasma samples which are accurately measured out by means of the calibrated tip-flush out-burette* technique of Seligson[28] and the decantation principle of Goldenberg.[29] This eliminates all pipetting and diluting to mark except for the addition of the sodium hydroxide and glucose oxidase and sulfuric acid solutions which are added with syringe pipettes.[30] The colorimetric readings are made with a Klett-Summerson photoelectric colorimeter using a No. 42 filter. The application of these general principles to routine biochemical determinations of substances other than glucose will be discussed in detail in subsequent publications.

In the event of emergency procedures, e.g., diabetic coma, the incubation time for both standards and unknowns may be shortened to 10 minutes at 37° C. and the remainder of the procedure remains unchanged. This time-saving inovation will still maintain the average error below ± 10 per cent of the "true" value as was determined in runs using glucose standards.

Another experimental finding of some importance for the routine clinical analysis of blood glucose is the fact that the addition of sodium fluoride (2 mg. per milliliter of blood) *does not inhibit* enzyme activity but does prevent glycolysis for 48 hours when the sample is kept refrigerated. However, the use of thymol as a preservative *does inhibit* glucose oxidase enzyme activity and its use as a preservative should be avoided. In practice this means that it is possible to collect a large number of blood samples in tubes containing fluoride for glucose analysis, e.g., glucose tolerance tests and carbohydrate studies, over a 24-hour period or longer and then perform all the determinations simultaneously.

From a practical viewpoint, the question of inhibitory substances which may be present in biologic fluids and thus prevent the reaction from proceeding to completion is of the greatest significance. It was mentioned in the introduction that such substances are present in significant, but variable amounts, in serum (or plasma) and to an even greater extent, i.e., over 80 per cent, in urine.[25] Many substances have been tested and have been found to have little or no effect on the enzymatic oxidation of glucose by glucose oxidase. These include a large number of common drugs and medications,[23] cyanides, hydrogen sulfide, azides and narcotics,[19] Mercuhydrin, sulfathiazole, bromides, streptomycin, fluorides, chlorides, and urea.[25] Of the substances normally present in urine which may account for its marked inhibitory effect on the glucose oxidase reaction, only ascorbic acid[23] and uric acid[25] have been mentioned. According to Teller[25] only 40 μg of the latter substance will cause a 50 per

*Burettes of this kind are obtainable from Kopp Laboratory Supplies, 1680 2nd Ave., New York 28, N. Y. (Clinalysis Apparatus), or from A. H. Thomas & Co., Philadelphia, Pa. (Seligson pipette).

cent loss of glucose. For routine glucose determinations he recommends a preliminary treatment with activated charcoal to remove inhibitory compounds prior to the analysis. This marked retarding effect of uric acid may also account for the differences between the results obtained by the direct analysis of plasma and those found for plasma zinc filtrates. It has been reported in the literature that Somogyi zinc filtrates contain practically no uric acid.[1]

The one experimental factor which was not thoroughly investigated in the present study was the effect of pH variation on the two enzyme systems. This was maintained at pH 7.0 with 0.005 M phosphate buffer (Sorenson). This pH value of the substrate gave stoichiometric readings over a range of values from 25 to 400 mg. per cent of glucose after acid addition to stop the enzymatic activity. These experimental findings, in addition to the fact that pH optimums of glucose oxidase and horse-radish peroxidase are 5.6 and 8.0 units, respectively, eliminated the necessity for a systematic investigation of this factor.

SUMMARY

1. A simple, rapid, quantitative, spectrophotometric method is presented for the microdetermination of heparinized plasma or blood glucose using glucose oxidase, a *specific* enzyme for beta D-glucose.

2. Systematic spectrophotometric studies were made of the various factors which may influence the optical density values obtained with a given glucose concentration for the two enzyme system. The factors studied (and their optimal values) were: (*a*) time of incubation (30 min.), (*b*) temperature of incubation (37° C.), (*c*) final sulfuric acid concentration (0.06 N), (*d*) time of reading after acid addition (5 to 30 min.), and (*e*) spectral adsorption curve (maximum at 395 mμ or No. 42 Klett filter). The concentration curve follows Beer's law over a range from 0.0125 to 0.200 mg. glucose (or 25 to 400 mg. per cent for a 1:20 zinc filtrate).

3. Attempts to apply the method directly to plasma (or serum) were unsuccessful because of the presence of variable amounts of inhibitory substances, e.g., uric acid and ascorbic acid. Since these are removed during preparation of Somogyi zinc filtrates, subsequent determinations were performed on such filtrates in comparison with the Nelson-Somogyi procedure. Oxalated plasma yields slightly lower (5 mg. per cent) results than heparinized plasma. Sodium fluoride (2 mg./ml.) does *not inhibit* the enzymatic reaction and can be used to prevent glycolysis where many samples are to be run.

4. Glycolysis is also minimized by the use of blood collected in siliconated tubes containing small amounts (100 μg) of heparin. This permits the plasma glucose analyses to be performed within a few minutes after drawing blood.

5. Comparative data (in mg. per cent) with the glucose oxidase (G.O.) and the Nelson-Somogyi (N.S.) methods were performed on fasting plasma obtained from (*a*) normal individuals (33), N.S. (mean ± s.d.) = 99.5 ± 8.8, G.O. (mean ± s.d.) 90.9 ± 7.9; (*b*) nondiabetic patients (36), N.S. (96.8 ± 9.1), G.O. (87.1 ± 8.8); and (*c*) diabetic patients (36), N.S. (168.4 ± 56.7), G.O. (147.5 ± 51.9).

6. Both the G.O. and N.S. methods have a ± 2 per cent error as determined by triplicate analyses on 15 standard serum samples. The normal range for hospitalized nondiabetic patients is 69 to 105 mg. per cent which is approximately the same as that obtained with the Benedict method. For routine analyses, a rapid simplified procedure is described for use with a photoelectric colorimeter which employs syringe pipettes and a decantation step.

7. Since the G.O. procedure measures "true" glucose, differences between the N.S. and G.O. methods constitute a measure of the filtrate nonglucose reducing substances or "saccharoids." Saccharoid mean ± s.d. for the 3 groups studied are (*a*) 8.6 ± 3.3, (*b*) 9.9 ± 2.8, and (*c*) 20.9 ± 8.8. In no instance did the saccharoid values of group *a* or *b* exceed 16 mg. per cent, whereas 50 per cent of the diabetic patients gave higher results including those whose blood sugar levels were within normal limits. The nature of the saccharoids present in diabetes is under investigation.

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AN INSTRUMENT AND METHOD FOR AUTOMATIC, RAPID, ACCURATE, AND SENSITIVE TITRATION OF CHLORIDE IN BIOLOGIC SAMPLES

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THE instrument and method to be described for titration of chloride with silver ion are based on established principles of coulometric generation of reagent and of amperometric indication of the end point.[1] A constant direct current is passed between a pair of silver *generator* electrodes in the generator (coulometric) circuit, causing release of silver ion into the titration solution at a constant rate. The end point is indicated after all chloride has been precipitated by the increasing concentration of free silver ion which causes a rising current to flow through a pair of silver *indicator* electrodes and a Meter-Relay in the indicator (amperometric) circuit. At a pre-set increment of indicator current the relay is actuated, stopping a timer which runs concurrently with generation of silver ion. Since the rate of generation of silver ion is constant, the amount of chloride precipitated is proportional to the elapsed time.

MATERIALS AND METHODS

Operation of the Instrument.—A view of the instrument is shown in Fig. 1 and a circuit diagram in Fig. 2. The sample is raised into position, the Titration Switch is turned to the No. 1 position (starting the stirrer), and the running time meter is reset to zero. The adjustable pointer of the Meter-Relay is set 10 μa above the reading of the indicating pointer (initial indicator current). The Titration Switch is then turned to the No. 2 position, starting both the timer and the generation of silver ion. At the end point the indicating pointer reaches the setting of the adjustable pointer, closing the switch contacts they carry, and thus actuating the relays which shut off timer, generator current, and stirrer. The elapsed time is read to the nearest 0.05 second.

Electrochemical Reactions at the Electrodes.—The voltage impressed across the *indicator* electrodes is 0.25 to 0.14 volt (values below 0.25 v. are due to voltage drop across the Meter-Relay signal coil, Fig. 2, c, with indicator current flow up to 40 μa). In this voltage range, free silver ion is reduced at the indicator cathode at a maximum rate for a given concentration, and silver ion is simultaneously released at the indicator anode. Only negligible amounts of other substances in the solution can undergo oxidation or reduction at the indicator electrodes. Thus, the initial current is usually less than 2 μa. The Titration Rate Switch provides a choice of 3 rates of titration: low, medium, and high, representing generator current of about 1.5 Ma, 6 Ma, and 24 Ma, respectively. The current efficiency of silver ion generation was found to be 100 per cent (the ratio of Faraday's constant to

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the electrochemical equivalent determined by titration of chloride standards and precise measurement of generator current was 100.7 per cent at low, 99.9 per cent at medium, and 100.0 per cent at high). This result shows that the only electrochemical reaction occurring at the generator anode is oxidation of metallic silver to silver ion, none of which is lost by reduction at the generator cathode (at this cathode hydrogen ions of the solution are reduced to hydrogen gas, since the voltage between the *generator* electrodes is 1 to 2 v.).

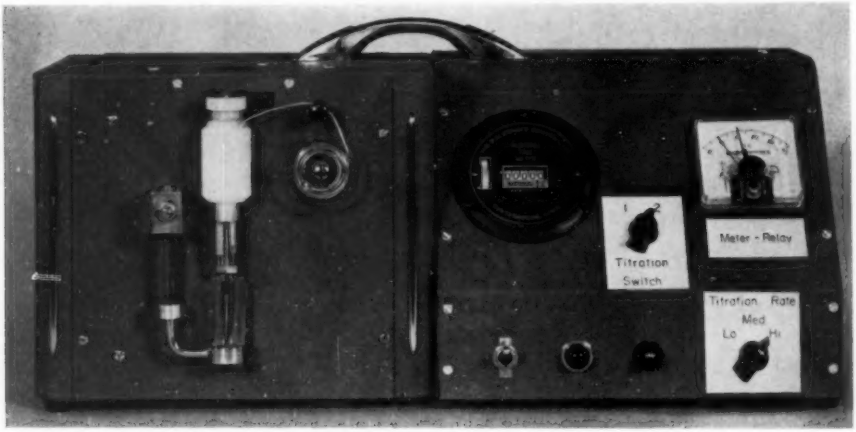

Fig. 1.—A front view of the instrument. The titration vial is in the lowered position. The indcator electrode pair is on the left and perpendicular to the generator electrode pair on the right, to avoid induced current. The generator anode on the right is continuous with a reserve supply of silver wire on the spool. The indicating pointer of the Meter-Relay is at zero, the adjustable pointer at 10 microamperes. The adjustable pointer is moved by means of the knob on the Meter-Relay. The stirrer turns at 700 r.p.m., driven by a phonograph turntable motor.

Conditions of Titration.—(1) The ranges of chloride content in the sample are, preferably: at low current titration, 0.25 to 3 μEq (net titration time about 64 sec./μEq); at medium current, 1 to 12 μEq (net time about 16 sec./μEq); at high current, 4 to 50 μEq (net time about 4 sec./μEq). (2) Total volume in the vial should be about the same for a group of analyses (± 10 per cent); about 2 ml. at low current and 2 to 5 ml. at medium or high. (3) The titration medium contains 0.1 N HNO_3, 10 per cent (1.75 M) acetic acid, and 25 mg. per cent gelatin as final concentrations (within ± 10 per cent). (4) Gelatin reagent is added on the day of titration, one drop per milliliter of solution in the titration vial. The gelatin reagent is prepared in large volume, divided into small portions and refrigerated; a fresh portion is used each day. It contains 600 mg. per cent gelatin, 10 mg. per cent thymol blue (to confirm presence of gelatin and acid in the vial), and 10 mg. per cent thymol (for preservation). (5) The temperature of the solution in the vial should be 20 to 30° C. A slight but increasing error is incurred with departure from these limits in either direction.

Typical Preparation of Plasma and Urine Samples.—Pipette into titration vial 0.1 ml. of plasma or urine, about 4 ml. of 0.1 N HNO_3, 10 per cent acetic acid, and 4 drops of gelatin reagent. Titrate at high current. Smaller volumes, or aliquots of dilutions of larger volumes, may be titrated.

Typical Preparation of Blank Samples.—For titration at high or medium current, deliver into titration vial about 4 ml. of 0.1 N HNO_3, 10 per cent acetic acid, and 4 drops gelatin. For titration at low current, use half these amounts.

Typical Preparation of Standard Samples.—The amount of chloride standard is chosen to give a titration time of about 60 seconds. Exact volumes of NaCl in 0.1 N HNO_3 and 10 per cent acetic acid are pipetted into titration vials, and gelatin is added. Typical amounts are: 4 ml. of 4 mEq./L. at high current; 4 ml. of 1 mEq./L. at medium current; and 2 ml. of 0.5 mEq./L. at low current.

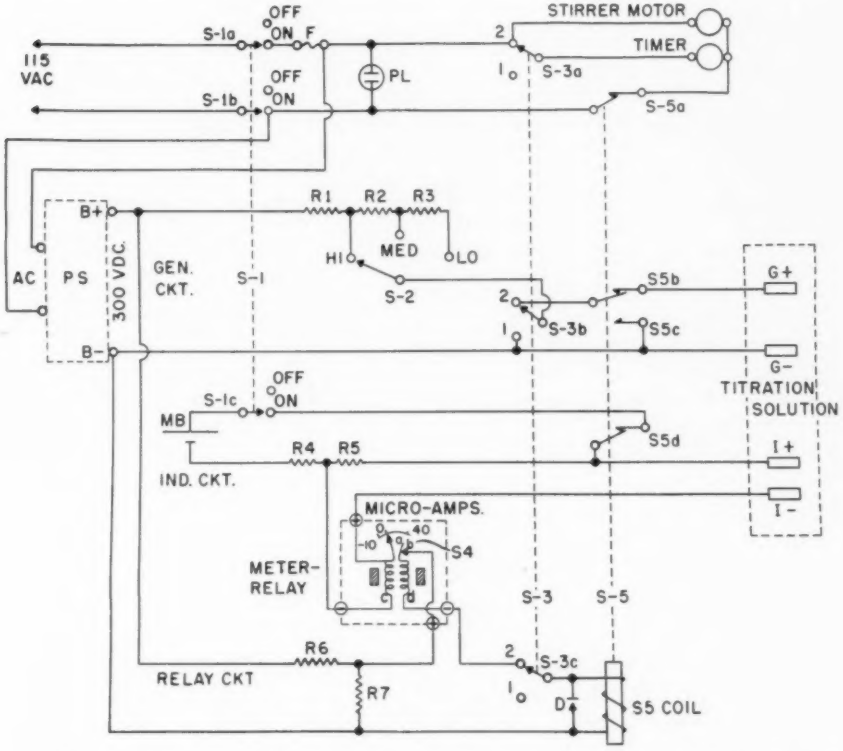

Fig. 2.—Circuit diagram of automatic titrator. Circuit is shown during titration at high current, before shut-off; S-1 is On; S-2 is at Hi; S-3 is at No. 2 (titrate) position; S-4 and S-5 contacts are in normal position (relays not energized).

S-1 Main Switch
S-2 Titration Rate Switch
S-3 Titration Switch
S-4 Relay of Meter-Relay
a. Moving Contact, carried on indicating pointer
b. Fixed Contact, carried on adjustable pointer
c. Signal Coil, 2,800 ohms, including damping resistor
d. Lock Coil of Relay Circuit, 100 ohms
S-5 Relay, 3 PDT (Potter-Brumfield No. KCP 14)
G+ Generator anode
G- Generator cathode
I+ Indicator anode
I- Indicator cathode

R1. 12.5-K, 50 w.
R2. 35-K, 10 w.
R3. 150-K, 2 w.
R4. 220 ohms, 1 w.
R5. 50 ohms, 0.5 w.
R6. 8-K, 10 w.
R7. 4-K, 5 w.

MB Mercury battery, 1.34 v.

F Fuse, 3 amperes

PL. Pilot light, NE 51
D. Germanium diode, 1N 96
PS Direct current power supply
B+ and B- Positive and negative poles of D. C. supply (about 300 v.)

(Meter-Relay, Model 261-C, Assembly Products, Inc., Chesterland, Ohio; D. C. Power Supply, closely regulated, No. 3-70X, Dressen-Barnes Corp., Pasadena, Calif.; Timer, Type 640E, R. W. Cramer Co., Centerbrook, Conn.)

If preferred, the standard to be titrated along with the usual plasma and urine samples may be 0.1 ml. of 160 mEq./L. NaCl in distilled water.

Calculation of Chloride Content.—With each day's unknowns, a few blanks and standards are titrated. The timer reading is designated as "gross sec." "Net sec." of standards or unknowns is obtained by subtracting the average gross seconds of blanks.

$$K = (\text{standard factor in } \mu\text{Eq/sec.}) = \frac{(\text{ml. of std.}) \times (\text{conc. of std. in mEq./L.})}{(\text{av. net sec. of standard})}$$

$$(\text{conc. of unknown in mEq./L.}) = K \times \frac{(\text{net sec. of unknown})}{(\text{ml. of unknown})}$$

RESULTS

Relation of Titration Time to Amount of Chloride.—Precise volumes of standard NaCl solution, of urine, and of plasma, representing varied levels of chloride, were titrated automatically at low, medium, or high generator current.

TABLE I. STATISTICS OF LINE OF REGRESSION OF GROSS SECONDS FOR TITRATION ON AMOUNT OF CHLORIDE

	CHLORIDE STANDARDS (NaCl)			URINE	PLASMA
Generator current	Low	Medium	High	Medium	High
No. of Cl levels*	7	7	8	7	5
No. replic. each level	6	4	4	4	4
Av. blank ± S.E. (sec.)	7.48 ± 0.04	4.55 ± 0.08	1.44 ± 0.03	4.69 ± 0.04	1.46 ± 0.04
Intercept ± S.E. (sec.)	7.94 ± 0.25	4.88 ± 0.08	1.43 ± 0.09	5.50 ± 0.01	1.31 ± 0.16
S.D. of single analysis from line (sec.)	0.84	0.22	0.41	0.20	0.36
S.D. of slope (% of mean)	0.21	0.06	0.12	0.06	0.32
Reciprocal of slope (μEq/sec.)	0.01563	0.05893	0.2559	0.5983†	2.506†
μEq/sec. factor of reference sample‡	0.01549	0.05858	0.2557	0.5908†	2.505†

*Microequivalent of chloride ranged between 0.25 and 3 at low current, 1 to 12 at medium, and 2.5 to 50 at high; aliquots of plasma ranged between 0.025 and 0.20 ml. and of urine between 0.01 and 0.12 ml.

†Microliter of urine or plasma per second.

‡Reference sample is that nearest 60 seconds titration time (see text).

Blank samples were also titrated. The results are summarized in Table I. The line of regression of gross seconds on amount of chloride was calculated in each case by the method of least squares, using average values at each level, and excluding blank samples. In each case, the line has a positive intercept on the time axis. The intercept gives the best estimate of the true blank time, which comprises a small component due to traces of silver-combining substances in the reagents, plus the "overtitration time." The overtitration time is that required after the equivalence point until the Meter-Relay is tripped by the rising indicator current (rise of indicator current of 10 μa above the initial value). The average measured blank differs statistically from the intercept value only in the case of the urine sample, and even here the difference is small.

The relation of gross seconds for titration to amount of chloride is linear to a high degree of precision in all 5 samples, as shown by the small standard deviations of the slopes of the lines: between 0.06 to 0.32 per cent of the mean values of each. The linear relation also has been found at longer titration times up to above 500 sec. at low or medium current (within 0.3 per cent of expected

values) and to as short as 5 sec. at high current (within 1 to 3 per cent). At high current, titration times above 200 sec. may be erratic because of the large amount of precipitate.

The variation of replicates at any chloride level is small: the coefficients of variation of the subgroups of Table I ranged from 0.04 to 1.5 per cent and were below 0.64 per cent for titration times of 40 sec. or more. In Table I, the standard deviation of a single analysis from the line gives a measure of the total variability of all analyses in each group, including all levels, and is between 0.20 and 0.80 sec. for the 5 groups. Because of the precise linearity and the correspondence of measured blank with intercept, the factor of proportionality (μEq/sec.) between amount of chloride and titration time can be accurately determined from the net titration time of a single reference standard (selected to give a titration time of about 60 sec.). The standard factor so obtained corresponds closely to that obtained from the least squares line which includes all the chloride levels (expressed as reciprocal of slope in Table I). From day to day the standard factor has shown no greater variability than on a single day.

Recovery of Chloride Added to Plasma and Urine.—Pooled human plasma was dialyzed against 0.02 M phosphate buffer at pH 7.4. The titration value of the dialyzed plasma was 0.56 mEq./L. Titration was performed in duplicate at high current on 9 combinations of addition of 5, 10, or 20 μEq NaCl to aliquots of dialyzed plasma of 0.05, 0.10, and 0.20 ml. The recovery of the added chloride was between 99.7 and 100.2 per cent (average of duplicates).

Low-chloride urine (1.69 mEq./L.) was obtained from a subject on a low-salt diet and a similar recovery experiment performed. The per cent recovery was between 98.7 and 101.3 per cent (over-all average of 100.1 per cent) for 4 levels of added chloride (2 to 8 μEq) added to 0.1 or 1.0 ml. of urine.

Effect of Plasma Protein on Chloride Titration.—Titration of chloride in plasma without removal of protein results in a slight positive error not detectable in a recovery experiment since the original value is subtracted. A sample of dialyzed plasma (containing 4.3 Gm. per cent albumin, 2.7 Gm. per cent globulin) gave titration values of 0.86 mEq./L. before, and 0.26 mEq./L. after protein removal by precipitation with 0.8 N HNO_3. The difference of 0.60 mEq./L. represents the extent of combination of silver ion with protein. The average difference in titration values of 9 nondialyzed plasma samples before and after deproteinization averaged 0.60 mEq./L. ($\pm$ 0.13 mEq./L. standard error); the average protein content was 3.9 Gm. per cent albumin, 3.3 Gm. per cent globulin.

In addition to the effect described, the presence of excess protein may increase the over-titration time to an extent greater than that estimated from a blank sample, by slowing the rate of rise of indicator current at the end point. Prolongation of the overtitration time of about 0.5 sec. was produced by added plasma protein of the following concentration in the titration solution: 100 mg. per cent at low current titration; 200 mg. per cent at medium and 1,200 mg. per cent at high current. The error is approximately proportional

to protein concentration, and is negligible in the case of direct dilution of plasma or urine (except for occasional urines with high-protein and low-chloride concentration).

Titration of Chloride in Tissue Extracts.—Depending upon the tissue and the method of preparation of the extract or digest, various amounts of sulfhydryl or sulfide groups may be produced which combine with silver ion, giving erroneously high values for chloride. The presence of these groups can be shown by their characteristic odor at acid pH, and quantitatively by addition of H_2O_2 to a solution at alkaline pH; these groups are oxidized and no longer combine with silver ion whereas chloride is unaffected. Even after dry ashing with Na_2CO_3 at 480° C. for 15 hours, some of such groups remain (probably sulfide). In a limited number of carefully controlled studies, no error resulted from simple water extraction (immersion of 1 mm. thick pieces in water at room temperature for 24 hours, with mixing); a large error resulted from alkaline digestion (dissolution in 0.1 N NaOH at 100° C.). Further studies on tissues are being conducted and will be reported later, but the following summarized data on frog gastric mucosa are illustrative. Methods were controlled by an isotope dilution technique using Cl^{36}; the relative amounts of silver ion required are expressed as a percentage of the titration value of the simple water extract (representing mucosal chloride content of 65 mEq./Kg. wet weight):

(a) dry ashing of water extract = 100.1 per cent
(b) H_2O_2 oxidation of (a) = 99.5 per cent
(c) alkaline dissolution of mucosas at 100° C. = 133.3 per cent
(d) H_2O_2 oxidation of (c) = 101.5 per cent
(e) dry ashing of (c) = 110.9 per cent
(f) H_2O_2 oxidation of (e) = 99.2 per cent

Comparison With Extrapolated End Point of Titration Curve.—The standard method of determining the equivalence point in an amperometric titration has been by graphing the titration curve and extrapolating the terminal linear portion of the rising indicator current after the equivalence point to intersect the value of initial current.[2] In a series of samples including standards, plasma, urine, and tissue extracts, net titration times were determined simultaneously by the present automatic method and by the extrapolated end point method (the entire titration curve was recorded in this series). The differences between the two methods in 200 individual samples averaged ± 0.14 second. The algebraic differences showed the automatic method to average lower by an insignificant amount (0.008 second).

DISCUSSION

In comparison with commonly employed methods for chloride which depend upon colorimetric titration[3] the present procedure has advantages of greater simplicity, rapidity and sensitivity, of the wide range of concentrations of

chloride which can be determined accurately in a single aliquot, of the avoidance of deproteinization or digestion, color interference, and operator variability in recognition of the end point. In comparison with electrometric procedures previously described[1, 4-8] the present method has the advantages of accurate and consistent and automatic detection of the end point in a wide variety of biologic samples.

The use of an amperometric end point to obtain automatic shut-off at a pre-set increment of indicator current, requires that the titration curves in varied samples be highly reproducible. In the course of the present study, indicator electrodes of platinum, silver-plated platinum, gold,[4-8] and tantalum were tried, but consistent results were obtained only with the combination of a silver anode and a silver or gold cathode. Results were superior if both indicator electrodes were silver, and this combination has given consistent titration curves in the course of thousands of titrations of a variety of biologic samples. The Meter-Relay allows easy setting of the shut-off point at a constant increment of indicator current, despite differing initial currents. Data have been presented to show that the over-titration time is accurately determined by titration of a blank sample.

Some protein in the titration solution is required in order to obtain a smooth indicator current and to prevent reduction of AgCl, and this is supplied by 25 mg. per cent gelatin.[2] The presence of additional protein, however, may introduce some error, due to combination of silver ion with available sulfhydryl groups. Titration of plasma without deproteinization gave values which were erroneously high by 0.60 mEq./L. in plasma of approximately normal protein content. An average value in normal sera of 0.54 mEq./L. of SH was obtained by Weissman and associates,[9] using argentometric titration in ammoniacal solution, and sera of low-protein content gave values as low as 0.07 mEq./L.[10] The available SH determined by their method was about 0.10 mEq./L. per Gm. per cent of albumin and 0.04 mEq./L. per Gm. per cent of globulin.[9] With the present method, the error in titration of plasma without deproteinization is unimportant for most purposes.

SUMMARY

An instrument and method are described which provide accurate, rapid, and automatic analysis of chloride in biologic samples, by electrometric titration with silver ion. Titrations times of 20 to 60 seconds per sample have a standard deviation of about 0.5 per cent of the mean value, and shorter times down to 5 seconds have only slightly greater variability. The method is sensitive, measuring accurately as little as 0.25 μEq of chloride. Operation of the instrument is simple and can be performed by personnel with little experience. Preparation of plasma, urine, or water extracts of tissue for titration requires only addition of reagent.

We gratefully acknowledge the technical assistance of Harry L. Morrison and the assistance of Dr. Max Halperin in the statistical analysis.

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A SIMPLIFIED SEMIAUTOMATIC BIO-ASSAY APPARATUS

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WITH THE TECHNICAL ASSISTANCE OF GEORGE H. ARMSTRONG

PHARMACOLOGIC assays on isolated preparations have become greatly simplified by the recent introduction of automatic and semiautomatic types of apparatus.[1-4] However, the above equipment is either very complex in design or its essential components are expensive or not readily available.

The present apparatus has been designed primarily for cathechol amine determinations utilizing the uterus assay procedure of Garcia de Jalon and associates.[2, 5]

MATERIALS AND METHODS

The following materials were used in assembling the semiautomatic bioassay apparatus: 1 fixed-interval repeat-cycle timer* with a ½ r.p.m. synchronous motor, 4 adjustable cams, and 4 double-throw snap switches; 3 Westinghouse solenoids†; one 18-volt pilot light bulb with socket assembly; 2 banana plugs; insulated wire; polyethylene and thin-walled rubber tubing (5 mm. diameter).

An isolated organ bath containing a rat uterus was set up according to the classical arrangement. Two Mariotte bottles (1- and 2-liter capacity, containing modified Tyrode's solution‡ with carbachol and without carbachol, respectively) were placed at a convenient height above a 2 ml. isolated organ bath. Two small inlet tubes fitted in the rubber base of this bath were connected to the Mariotte bottles by polyethylene tubing except at the points where the solenoid clamps were applied. Thin-walled rubber tubing was used at the points of clamping. Air was bubbled by means of a hypodermic needle introduced through the rubber base of the bath; the isolated tissue was attached to this needle. The fluid level was kept constant by a suction tube as suggested by Gaddum and Lembeck.[2] A thermoregulated water bath was used to maintain the desired temperature for the solutions and isolated tissue.

Delivery of the solutions is controlled by 2 electromagnetic clamps connected with snap switches No. 1 and 2 of the cycle timer (Fig. 1). The snap switches are operated by the cams of the fixed-interval repeat-cycle timer. Each of the 4 sets of cams consists of 2 disks side by side on the timer shaft. Half of the circumference of each disk has a smaller diameter than the remainder. One disk can be rotated manually from the other member of each pair and locked in position; this adjustment forms a notch that determines the time interval of the release of the snap switch. In this position of the cams, the current to the electromagnetic clamps is off when the circuit is connected to the snap switch lead labeled "normally open." The rate of flow to the tissue bath was controlled by an adjustable Hoffman clamp.

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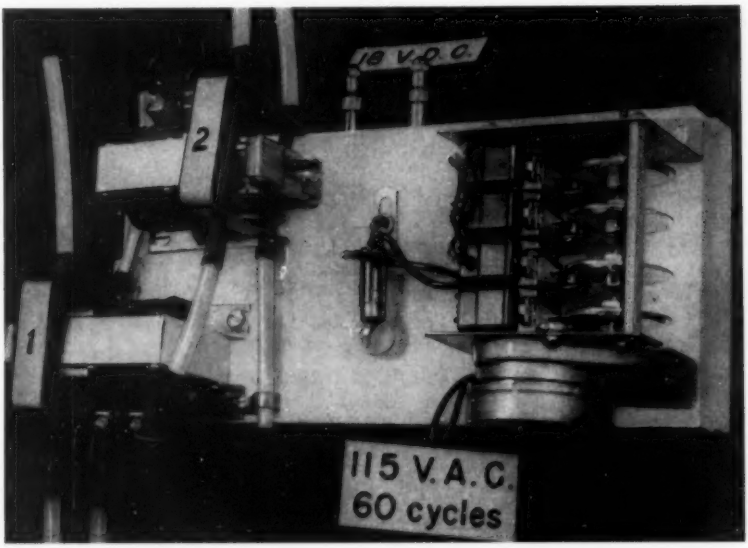
*Series 5800 obtained from Haydon Manufacturing Co., Torrington, Conn.

†Part No. Q-9428.

‡Modified Tyrode's solution as prepared by Gaddum and Lembeck.[2]

A third electromagnetic clamp, not shown in Fig. 1*A*, was connected to the lead labeled "normally open" of snap switch No. 4 and controlled overflow suction time. Rubber tubing was attached to the side arm of a T-tube inserted in the suction line; the free end of the tubing was passed through this clamp.

A.



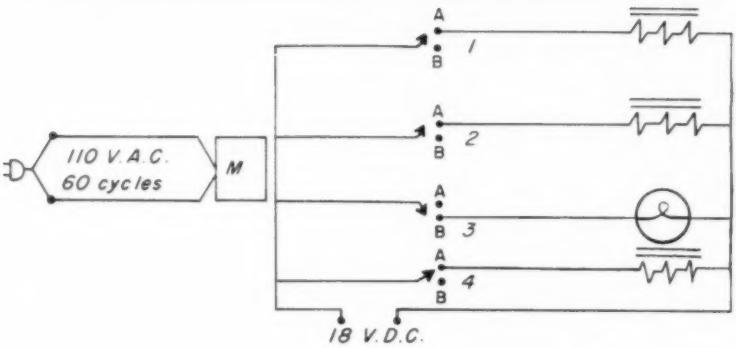


Diagram of the electrical circuit

B.

Fig. 1.—A simplified semiautomatic apparatus for bio-assay. In *B*, diagram of the electrical circuit, M = synchronous motor; A = "normally open" and B = "normally closed" snap switch leads, respectively.

An 18-volt pilot light bulb was connected with the lead of snap switch No. 3 labeled "normally closed." The lamp was on for approximately 5 seconds and signaled the manual addition of standard or unknown samples to the tissue bath.

RESULTS

Fig. 2 serves to illustrate the sequence of events in a 2-minute cycle as controlled by the described apparatus. An isolated uterus was suspended in modified Tyrode's solution with and without added carbachol. From a to b (5 seconds), 2 ml. of Tyrode's solution containing carbachol entered the organ bath; from c to d (5 seconds), the tissue was washed with 10 ml. of Tyrode's fluid; from e to h (30 seconds), the suction line was off; from f to g (5 seconds), the lamp was on signaling the addition of standard or unknown samples; at aa, the cycle was repeated.

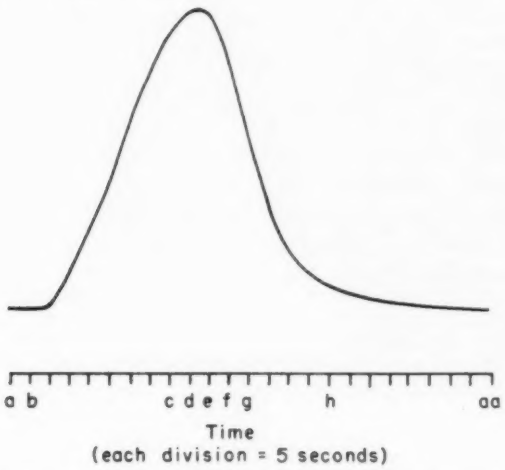


Fig. 2.—Contraction of the isolated uterus as influenced by the semiautomatic bio-assay apparatus. A 2-minute bio-assay cycle as controlled by the proposed apparatus. Upward tracing, contraction of the uterus; horizontal scale, time (each division equals 5 seconds).

DISCUSSION AND SUMMARY

A semiautomatic bio-assay apparatus was described; the essential components are readily available and it is simple in design, relatively inexpensive, and easily assembled. Possible applications of this equipment include the bio-assay of: epinephrine, norepinephrine, antihistaminic compounds, curarelike drugs, and spasmolytic agents.

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ADDENDUM

(Abstract of Reference 5.)

Sensitive and New Method of evaluation of adrenaline in isolated rat uterus

The present procedure is based on the adrenaline inhibition of spontaneous motility of isolated rat uterus (Knaus and Clark, 1925), on the evaluation of ergot by Broom and Clark (ref. not cited), and on the inhibition of spontaneous motility as reported by Beauvillain (1943) using a solution with the following composition: sodium chloride, 9 Gm.; potassium chloride, 0.42 Gm.; calcium chloride, 0.06 Gm.; magnesium chloride, 0.005 Gm.; sodium bicarbonate, 0.5 Gm.; glucose, 0.5 Gm.; distilled water, quantity sufficient for 1,000 ml.

A 75 ml. oxygenated tissue bath at 29° to 30° C. was used. The spontaneous uterine movements were inhibited after several washings at this temperature. Contractions were induced with acetylcholine (1:1 million to 1:3 million); the first 4 or 5 contractions were discarded. The duration of the acetylcholine contraction was 1 to 1.5 minutes and sometimes it was followed by several smaller contractions. The test was started once the uterus responded with contractions of the same height after optimal doses of acetylcholine. Following each induced contraction by acetylcholine, the bath was washed. Addition of adrenaline did not change the base line; 1 minute or 1.5 minutes later acetylcholine was added. The height of the contraction obtained was dependent exclusively on the dosage of adrenaline used. At high doses of adrenaline the acetylcholine-induced contraction may be abolished. An unknown adrenaline sample can be compared with a known standard using the above procedure.

Garcia de Jalon and co-workers cite the following advantages in the proposed method: sensitivity to 0.01 μg adrenaline when using a 75 ml. bath; stability of response after repeated assays; easy to perform; and low cost.

DIFFUSION OF PROGESTERONE AND DESOXYCORTICOSTERONE INTO NITROCELLULOSE (LUSTEROID): A POTENTIAL SOURCE OF ERROR IN ULTRACENTRIFUGATION

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WHEN an aqueous solution of progesterone is kept in a tube made from "Lusteroid" (cellulose nitrate), part of the steroid disappears from the solution. This was observed when aqueous solutions of C^{14}-tagged steroid hormones were subjected to ultracentrifugation in Lusteroid tubes.[1] The interaction of progesterone with the tube wall was considerably reduced when albumin was present in the solution, presumably by competitive binding of the steroid to the protein. No decrease in concentration was detected when cortisol-4-C^{14} was kept in contact with the nitrocellulose plastic.[1]

The mechanism of this interaction between steroid and nitrocellulose was investigated since it is an uncommon reaction of a Δ^4-3-ketosteroid hormone. It is of considerable practical significance in all studies where small quantities of certain steroid hormones are handled in nitrocellulose plastics as, e.g., in high-speed centrifugation or ultracentrifugation.

MATERIALS AND METHODS

Steroid Solutions.—Properties and purification of the progesterone-4-C^{14} used (1 μg = 8,400 c.p.m.) have been described previously.[2] A solution in water was prepared containing 0.55 μg per milliliter. The nonradioactive steroids,* after several recrystallizations from aqueous and water-free alcohol, had the melting points reported in the literature. They were dissolved in phosphate buffer of pH 7.6, $\mu = 0.1$, prepared from reagent-grade sodium salts and deionized distilled water. Details of the preparation of the aqueous steroid solutions and of their determination with the Beckman DU spectrophotometer have been outlined before[3]; progesterone, desoxycorticosterone, and cortisol were measured at their absorption maxima at 249, 249, and 248 mμ, respectively.

Kinetics of the Steroid-Lusteroid Interaction.—Five-tenths milliliter of an aqueous progesterone-4-C^{14} solution (0.55 μg per ml.) was placed into each of a number of Lusteroid tubes (60 mm. long, 12.5 mm. inside diameter, round bottom).† In all experiments the Lusteroid tubes, which had not been used before, were thoroughly cleaned with a detergent solution and rinsed with distilled water. The 0.5 ml. steroid solution covered a wall area of

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*Progesterone and desoxycorticosterone were kindly provided by Ciba Pharmaceutical Products, Inc., Summit, N. J., and cortisol by The Upjohn Company, Kalamazoo, Mich.

†Lusteroid tubes obtained from Specialized Instruments Corporation, Belmont, Calif., as accessories to the Model E Spinco ultracentrifuge, rotor J.

approximately 2.3 square cm. The tubes were kept at 4° C. and 0.1 ml. samples were withdrawn at the intervals indicated in Fig. 1, Curve I. Quadruplicate determinations of the C^{14} radioactivity were made using gas-flow-counter techniques described previously.[1, 2]

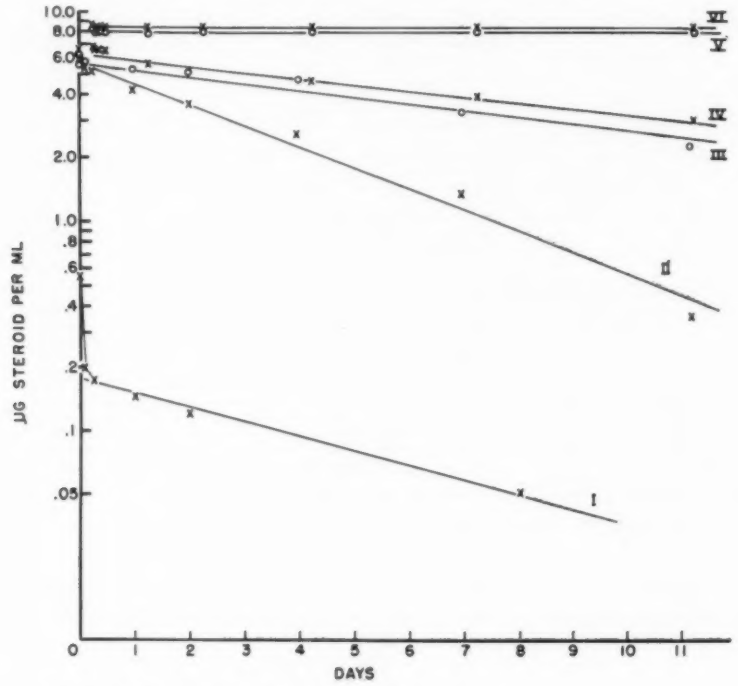

Fig. 1.—Diffusion of steroids into Lusteroid.
Curve I: 0.55 μg progesterone-4-C^{14} per ml.; 4° C.*
Curve II: 6.36 μg progesterone-C^{12} per ml.; 24° C.
Curve III: 6.12 μg progesterone-C^{12} per ml.; 4° C.
Curve IV: 6.69 μg desoxycorticosterone per ml.; 24° C.
Curve V: 8.54 μg cortisol per ml.; 24° C.
Curve VI: 8.20 μg cortisol per ml.; 4° C.
Solvent: phosphate buffer pH 7.6, $\mu = 0.1$.
*Solvent: water; surface area of Lusteroid: 2.3 square cm.

In the studies employing nonradioactive steroids, 15 ml. of the steroid solutions in phosphate buffer, at the concentrations given in Figs. 1 and 2, and in Table I, were put into 25 ml. glass-stoppered Erlenmeyer flasks. Four hollow Lusteroid cylinders of 12.5 mm. height and 12.5 mm. inside diameter, obtained by dissecting the 50 mm. long straight part of a Lusteroid tube, were placed into each of the flasks. A total surface area of approximately 40 square cm. was thus provided which was completely covered by the 15 ml. of the steroid solutions. Microscopic observation indicated that the Lusteroid material had a very smooth, glossy surface; a few occasional scratches did not appear of sufficient magnitude to change the surface area calculated from the macroscopic dimensions. Controls were set up in the same way except that 15 ml. of steroid-free phosphate buffer surrounded the Lusteroid rings. The solutions were placed on a shaking machine (100 strokes per min., 3 cm. extension with the experiments illustrated in Fig. 1, Curves II-VI; 120 strokes per min., 4 cm. extension with the experiments illustrated in Fig. 2) and kept at the indicated temperatures. At the

time intervals noted in Figs. 1 and 2, aliquot samples were removed, measured in the Beckman DU spectrophotometer and immediately returned to the original Erlenmeyer flasks. The steroid-free controls were used as blank solutions in the spectrophotometric measurements.

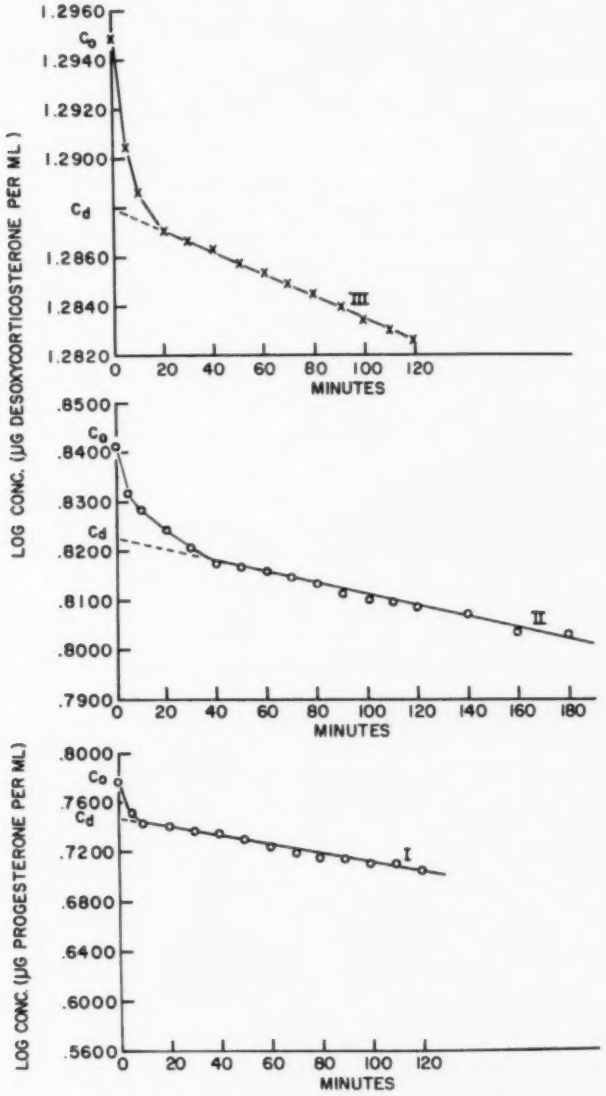

Fig. 2.—Diffusion of progesterone and desoxycorticosterone into Lusteroid.
Curve I: 5.99 µg progesterone per ml.; 26° C.
Curve II: 6.94 µg desoxycorticosterone per ml.; 26° C.
Curve III: 19.72 µg desoxycorticosterone per ml.; 25° C.
Solvent: phosphate buffer pH 7.6, $\mu = 0.1$.

For the chemical identification of the steroid which had diffused into the nitrocellulose, progesterone-C^{12} and progesterone-C^{14} solutions were exposed to Lusteroid in the described manner. After 6 days when the steroid concentration had decreased to 43 per cent of its original value, the nitrocellulose was removed from the solution, rinsed with water and 96 per cent alcohol to remove any surface-adsorbed steroid, scissored into small pieces, and extracted with 96 per cent alcohol at approximately 65° C. for 72 hours. Paper-chromatographic analysis in hexane-propylene glycol[4] using colorimetric[5] and radiometric[2] techniques failed to demonstrate any steroid compound except progesterone. It is concluded that the interaction of nitrocellulose with the $\triangle^4$-3-ketosteroids studied does not involve a chemical alteration of the steroid molecule.

TABLE I. INTERACTION OF PROGESTERONE AND DESOXYCORTICOSTERONE WITH LUSTEROID

STEROID*	CURVE IN FIG. 2	STARTING CONC. (C_0) μG/ML.	TEMP. (C.)	ADSORBED PER 40 CM.^{2} LUSTEROID (μG)	Å.^{2} PER ADSORBED MOLECULE†
P	–	6.11	29°	7.89	27
P	I	5.99	26°	5.75	36
D	–	6.94	29°	3.99	55
D	II	6.94	26°	4.40	50
D	III	19.72	25°	4.65	47
D	–	19.56	27°	4.35	51

*P = progesterone; D = desoxycorticosterone.
†Assuming adsorption in a monomolecular layer.

RESULTS AND DISCUSSION

As may be seen from Fig. 1, Curve I, the concentration of progesterone-4-C^{14} in the Lusteroid tubes first declined sharply, then gradually, and reached a level of less than 10 per cent of the original content after 8 days. This continued disappearance of the steroid from the solution suggested actual diffusion into the Lusteroid rather than adsorption onto the wall surface. This assumption was corroborated when it was noted that the major part of the Lusteroid-bound progesterone-4-C^{14} could not be washed off its surface with ethanol. Although a small fraction of the Lusteroid-fixed steroid could be eluted by rapid washing, the major portion of it was recovered from the ethanol-rinsed and dissected plastic only by repeated and prolonged extraction with hot ethanol.[1]

Essentially similar disappearance rates were obtained for nonradioactive progesterone and desoxycorticosterone when solutions in phosphate buffer were kept in contact with a Lusteroid surface for periods up to 11 days at 4° C. and at room temperature (Fig. 1). Solutions of cortisol of similar concentration showed practically no change under identical conditions of exposure to the plastic. The removal of progesterone and desoxycorticosterone by the nitrocellulose material approximated a logarithmic decline. In all experiments the extension of the "disappearance curve" to time zero did not coincide with the level of the original concentration but stayed significantly below that value.

When the rates of decrease of the steroid concentrations were measured over shorter periods of time at room temperature disappearance curves were obtained as exemplified in Fig. 2; data for additional experiments are given in Table I and elsewhere.[6] The curves showed a rapid initial decline in the steroid concentration followed by a slower rate of disappearance. The straight line

obtained by plotting log concentration versus time was extrapolated to time zero to give the concentration c_d. This value constitutes the initial steroid concentration (c_o) less that portion which disappeared rapidly from the solution during the first minutes; this quantity, $c_o - c_d$, is interpreted as being removed from the solution by adsorption to the Lusteroid surface. Assuming adsorption in a monomolecular layer, the area required per molecule was calculated to be approximately 32 Å.^{2} per molecule with progesterone and about 51 Å.^{2} per molecule with desoxycorticosterone. Tripling the concentration of desoxycorticosterone did not cause a detectable change of the surface area requirement per molecule of this steroid (Table I).

The spatial dimensions of progesterone constructed from Stuart-type models (Fisher Scientific Company; 1 cm. = 1 Ångström unit) and measured against an Ångström scale* were found to be approximately 12 to 13 Å. long, 6 to 7 Å. wide and 5 to 6 Å. thick in satisfactory agreement with those found by x-ray diffraction measurements of progesterone[7] and steroids in general.[8] If one assumes that the Δ^4-3-ketosteroids are adsorbed as a monomolecular layer, the space requirements observed (Table I) would indicate that the molecule is adsorbed on its smallest projected area.

The plastic tubes used (Lusteroid) consist of cellulose nitrate[9] which is known to have an affinity for ketones.[10] It is believed, therefore, that the carbonyl groupings of the steroids are responsible for the adsorption to the Lusteroid surface and for the subsequent disappearance. The kinetic analysis of the disappearance of the steroids from the solution shows that the first step involves a comparatively rapid fixation of an amount of steroid which corresponds well to a monomolecular layer of the size of the Lusteroid surface. This initial fixation is referred to as "adsorption"; it might well be understood as the starting condition for the second phase which is interpreted as "solid solution" achieved by molecular migration of the steroid ketones into the nitrocellulose. Chemical evidence shows that the progesterone molecule is not altered by the interaction with the cellulose nitrate. It is noteworthy that, assuming a monomolecular layer, the space requirement for progesterone on nitrocellulose (about 32 Å.^{2}) is less than for desoxycorticosterone (about 51 Å.^{2}), even at about threefold concentration of the latter steroid. Apparently, the additional hydroxy group at C_{21} prevents a closer packing in the adsorbed layer.

The rate of steroid migration into the nitrocellulose, calculated from the observed rates of disappearance from the solution, is not lower than about 1 Å. per 10 minutes. This is a minimal value, and its accuracy cannot be more than that of an order of magnitude.

SUMMARY

Progesterone and desoxycorticosterone, but not cortisol, disappeared from aqueous solutions when brought into contact with a nitrocellulose (Lusteroid) surface. Initially, a rapid fixation to the surface took place. Assuming the

*Photographs of this model seen from the 3 spatial directions against an Å. scale have been given in Figs. 7 to 9 in reference 6.

formation of a monomolecular layer of adsorption, the space requirement per molecule of progesterone and desoxycorticosterone was approximately 32 and 51 Å.^{2}, respectively. The rate of the subsequent migration into the nitrocellulose was first order for the steroids.

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COMPARISON OF THE EXTRACTION OF POTASSIUM AND SODIUM FROM LIVER TISSUE BY ACID AND WATER

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INTRODUCTION

THE extraction of electrolytes from tissues for measurement by the flame photometer has been based either on the nitric acid extraction method as employed by Lowry and Hastings,[1] or the dry ashing method as described by Hald.[2] Several reports have appeared in the literature recommending the extraction of chlorides,[3] and potassium[4, 5] from tissues by leaching with water. Although this procedure for potassium was reported to compare favorably with the dry ashing method,[5] the data would appear to require additional study. The extraction of sodium from tissues by water has been attempted,[6] but not reported. This also requires further investigation.

In the present study, a comparison has been made of the accuracy of potassium and sodium analyses of liver carried out by the alternative methods of extraction by water and by nitric acid.

MATERIALS AND METHODS

Mice of a highly inbred Swiss white strain were used. The animals were all of the same sex (male) and weight (20 ± 3 Gm.). Animals were sacrificed by cervical dislocation. Livers were removed in less than 30 seconds and blotted dry. These were then dropped into liquid nitrogen to avoid a change in weight of the tissue by evaporative loss of water. The extraction of potassium and sodium by water was carried out as follows: a known weight of this frozen tissue (usually 150 ± 50 mg.) was transferred to a screw-cap tube which contained 0.8 ml. of ion-free distilled water. The tissue was macerated by means of a blunt glass rod and the mixture brought to 5 ml. volume with ion-free distilled water. The tubes were then sealed with plastic caps and placed in a hot-water bath; the liquid was allowed to boil for 30 minutes. Material in capped tubes was stored in a refrigerator overnight after which it was allowed to come to room temperature and then centrifuged for 30 minutes at 3,500 r.p.m. The extraction of potassium and sodium by nitric acid was as described by Mudge.[7] In both methods aliquots of the supernatants were suitably diluted after adding $LiNO_3$ to a final concentration of 250 p.p.m. for sodium and 100 p.p.m. for potassium analysis. The analyses were made with a Baird Associates flame photometer, which employs the internal standard principle.[8]

In designing this experiment, the aim was to ascertain the following: (1) the reliability of each method by determining whether variation occurred within the same tissue

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sample; (2) the degree of variation between animals; and (3) a comparison of the means between the water extraction and the acid extraction method.

In order to test the variability between methods and livers, multiple samples of tissues from the same liver were used for both the water and acid extraction of potassium and sodium. In this experiment (Tables I and II) several samples of tissue were taken from the same liver. Half of these were prepared for potassium and sodium analysis by the water extraction method and the other half by the nitric acid extraction method.

TABLE I. POTASSIUM MEQ. PER KG.

A. Water Extraction

ANIMAL NO.	1	2	3	4	5	6	7
Sample A	83.8	82.8	81.8	83.7	84.7	81.4	84.3
B	83.2	83.6	84.8	84.1	83.6	82.8	82.0
C	83.9	84.7	82.3	84.0	82.4	81.6	81.4
D	81.4	82.7	83.9	82.0	84.9	83.3	83.2
Mean	83.1	83.5	83.2	83.5	83.9	82.3	82.7
S.E. ±	0.58	0.46	0.21	0.29	0.58	0.46	0.56

B. Acid Extraction

ANIMAL NO.	1	2	3	4	5	6	7
Sample A	82.7	82.7	84.3	86.3	84.4	81.2	84.2
B	82.8	83.5	83.8	84.2	81.4	84.1	82.2
C	83.3	80.0	81.8	84.2	83.5	82.0	81.5
D	81.9	82.3	82.3	83.2	83.2	83.6	83.2
Mean	82.7	82.1	83.1	84.5	83.1	82.7	82.8
S.E. ±	0.29	0.73	0.60	0.65	0.63	0.68	0.56

C. Analysis of Variance

SOURCE	SUM OF SQUARES	*d. f.*	MEAN SQUARES	*f.*
Livers	12.32	6	2.05	1.49
Methods	0.36	1	0.36	0.26
Interaction	7.23	6	1.21	0.88
Residual	58.30	42	1.38	
Total	78.21			

TABLE II. SODIUM MEQ. PER KG.

A. Water Extraction

ANIMAL NO.	1	2	3	4	5	6	7
Sample A	33.9	34.9	35.2	34.1	34.5	34.5	32.4
B	34.4	32.4	32.5	33.3	35.3	32.3	34.5
C	38.7	34.7	33.0	35.6	35.6	35.8	36.1
D	33.7	32.3	33.0	36.1	32.2	32.6	32.1
Mean	35.2	33.6	33.4	34.8	34.4	33.8	33.8
S.E. ±	1.2	0.7	0.6	0.7	0.8	0.8	0.9

B. Acid Extraction

ANIMAL NO.	1	2	3	4	5	6	7
Sample A	37.8	32.3	34.4	32.3	31.6	33.6	34.2
B	35.8	31.1	35.4	36.0	34.9	31.6	32.5
C	32.4	35.6	31.7	34.6	35.6	35.0	31.2
D	35.2	32.3	31.6	32.6	32.9	36.9	32.2
Mean	35.3	32.8	33.3	33.9	33.8	34.3	32.5
S.E. ±	1.1	1.0	1.0	0.9	0.9	1.1	0.6

C. Analysis of Variance

SOURCE	SUM OF SQUARES	*d. f.*	MEAN SQUARES	*f.*
Livers	19.30	6	3.22	1.05
Methods	2.57	1	2.57	0.84
Interaction	11.38	6	1.90	0.62
Residual	128.84	42	3.07	
Total	162.09			

In order to compare the sensitivity of each method an additional experiment was carried out in which duplicate liver samples of various weights were taken from 15 mice for analysis after extraction by either leaching with water or digesting with nitric acid.

RESULTS

A comparison of the two methods used for the extraction of potassium (Table I) and sodium (Table II) indicates that the water leaching method is sufficiently accurate to be acceptable for the analysis of liver tissue. An analysis of variance[9] (Tables I, C and II, C) comparing the water leaching method with the acid digestion method suggests the following: (1) there is no significant variability among the livers within the method of extraction, (2) no significant variability is noted with regard to the method of extraction, and (3) the interaction of liver tissue and methods contributes no significant variability.

TABLE III. ANALYSIS OF LIVER FOR POTASSIUM

	WATER EXTRACTION			ACID EXTRACTION		
ANIMAL NO.	WT. OF SAMPLE (MG.)	POTASSIUM (MG./ SAMPLE)	POTASSIUM (MEQ./KG.)	WT. OF SAMPLE (MG.)	POTASSIUM (MG./ SAMPLE)	POTASSIUM (MEQ./KG.)
1	82	0.269	83.9	72	0.235	83.3
2	102	0.340	85.1	106	0.351	84.9
3	110	0.362	84.2	138	0.448	83.1
4	124	0.413	85.2	135	0.444	85.3
5	124	0.417	86.0	134	0.443	84.7
6	131	0.429	83.8	134	0.437	83.4
7	134	0.444	85.5	154	0.502	83.5
8	164	0.539	84.1	160	0.527	84.3
9	165	0.549	85.2	155	0.499	82.5
10	184	0.601	83.6	192	0.643	85.7
11	186	0.604	83.1	134	0.474	85.4
12	186	0.599	82.3	154	0.509	84.5
13	197	0.653	84.8	178	0.584	83.9
14	208	0.685	84.3	186	0.605	83.2
15	216	0.716	84.8	120	0.392	83.7
Mean			84.4			84.1
S.E. ±			0.08			0.08

TABLE IV. ANALYSIS OF LIVER FOR SODIUM

	WATER EXTRACTION			ACID EXTRACTION		
ANIMAL NO.	WT. OF SAMPLE (MG.)	SODIUM (MG./ SAMPLE)	SODIUM (MEQ./KG.)	WT. OF SAMPLE (MG.)	SODIUM (MG./ SAMPLE)	SODIUM (MEQ./KG.)
1	82	0.076	40.2	72	0.063	38.0
2	102	0.080	34.2	106	0.089	36.7
3	110	0.085	33.9	138	0.115	36.3
4	124	0.090	31.4	133	0.089	29.2
5	124	0.100	35.1	134	0.097	31.7
6	131	0.102	33.9	134	0.112	36.4
7	134	0.091	29.4	154	0.128	36.2
8	164	0.134	35.4	160	0.127	34.7
9	165	0.125	32.8	155	0.109	30.6
10	184	0.127	30.0	192	0.136	30.7
11	186	0.157	36.7	134	0.109	35.3
12	186	0.139	32.5	154	0.106	36.2
13	197	0.155	34.2	178	0.140	34.1
14	208	0.167	34.9	186	0.151	35.4
15	216	0.181	36.5	120	0.089	32.4
Mean			34.1			34.3
S.E. ±			0.71			0.80

Sample size within the range used did not appear to be an important factor in either of these extraction methods. Both methods demonstrated equal sensitivity (Tables III and IV). Measurement of potassium in livers of 15 mice gave a mean and standard error of 84.4 ± 0.08 for samples extracted with water as compared with 84.1 ± 0.08 for samples digested with acid (Table III). The difference is of low significance (> 0.02 P < 0.05). It suggests that the methods may give slightly different absolute values but the means are so close that neither method need be preferred on this basis. A comparison of duplicate samples by both methods for sodium indicated almost identical means (Table IV). The mean and standard error with the water extraction method was 34.1 ± 0.71 while that for the acid method was 34.3 ± 0.08 ($P > 0.1$).

DISCUSSION

The extraction of electrolytes from tissues by leaching with water was first suggested by Heilbrunn and Hamilton.[3] Stone and Shapiro[4] reported complete removal of potassium from brain and muscle when this cation was extracted with water. Seibert and associates[5] compared water extraction and dry ashing in the analysis of potassium in liver and found the former method was reliable within 4 per cent. In a personal communication, Dr. E. Cotlove[6] suggested that sodium could be extracted from liver by leaching with water. Such a method had been used in Dr. Cotlove's laboratory for the analysis of electrolytes in rat muscle and gave results which were comparable with those obtained with previous methods.

An analysis of the data from the present study indicates that the water extraction method compares favorably with the acid digestion method in accuracy. In addition to this there are the following practical advantages of the water leaching method over the ashing and nitric acid digestion methods now in use: (1) rapidity (extraction and determination can be completed within the same day); (2) both potassium and sodium analysis can be performed by flame photometer after direct dilution; (3) the greater convenience of using water as compared to handling concentrated nitric acid; and (4) a minimum of equipment is necessary.

SUMMARY

1. A method for the extraction of potassium and sodium from tissue by leaching with water is described.

2. A comparison is made between the water leaching method and the nitric acid digestion method for the extraction of potassium and sodium from liver tissue. An analysis of the data with regard to the method of extraction indicates that the use of the water leaching method for the extraction of these cations compares favorably with the acid extraction method.

3. Practical advantages of the water leaching method are discussed.

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THE FLUORIMETRIC MEASUREMENT OF PLASMA EPINEPHRINE AND NOREPINEPHRINE CONCENTRATIONS IN MAN, MONKEY, AND DOG

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IT IS well established that the adrenal medulla releases increased amounts of epinephrine and norepinephrine in a wide variety of conditions which call for an unusual effort on the part of the body to perform work, to adjust to changes in its internal environment, or to resist threatened dangers. Many aspects of the regulation of epinephrine and norepinephrine secretion by the central nervous system, however, are as yet little understood. Experimental work in this area has been greatly handicapped by the scarcity of suitable methods for the accurate analysis of both central nervous system function and adrenal medullary activity. Within recent years improved methods for the systematic study of both emotional behavior and neurophysiologic mechanisms in the experimental animal have heightened interest in the search for specific chemical methods for the quantitative measurement of epinephrine and norepinephrine in biologic fluids. A method currently of considerable interest is that described by Weil-Malherbe and Bone[1] which employs a chromatographic separation of the catechol amines from other fluorescent plasma constituents and the differentiation of epinephrine and norepinephrine by fluorimetric procedures. Although several laboratories have reported success in using the method,[2-4] some concern over its validity continues to be expressed in the literature.[5] Also there is a particular need for data on which to base the interpretation of results obtained with low-hormone concentrations such as found in the peripheral plasma of human beings, monkeys, and dogs. The purpose of this report is to present the data obtained in a systematic evaluation of the Weil-Malherbe method, with the viewpoint of determining the suitability of the method for the measurement of epinephrine and norepinephrine in human, monkey, and dog plasma.

METHODS AND MATERIALS

Reagents.—

Sodium fluoride–sodium thiosulfate solution, NaF (2 Gm.) and $Na_2S_2O_3$ (3 Gm.) was dissolved in glass-distilled water to 100 ml. The solution was autoclaved and transferred to sterile ampoules, each containing about 6 ml.

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Sodium carbonate, a 0.5 N solution.

Sodium acetate buffered at pH 8.4. A 0.2 M solution of sodium acetate was passed over a column of Zeo-Karb 215 (Permutit Co., Ltd.) and adjusted to pH 8.4 by the addition of 0.5 N Na_2CO_3.

The Zeo-Karb column was prepared by repeated washings with 2 N HCl, water, and 4 per cent NaCl, the last being continued until the pH of the filtrate was about 6. Excess NaCl was flushed out with glass-distilled water.

Alumina, acid washed. Al_2O_3 (100 Gm.; Fisher Scientific, "for chromatographic analysis 80-200 mesh") was stirred with 500 ml. of boiling 2 N HCl for 20 minutes, filtered, and washed on the funnel with 500 ml. of hot 2 N HCl. It was then repeatedly washed by decantation with distilled water and dried at 300° C. for 3 hours.

Acetic acid, a 0.2 N solution.

Ethylenediamine dihydrochloride (Eastman Kodak Company, Organic), a 2 M solution.

Ethylenediamine (Eastman Kodak Company, Organic). Redistilled immediately prior to use.

Isobutanol (Merck & Co., Inc., "suitable for fluorimetric work"). Redistilled immediately prior to use.

Procedure.—

Collection of samples: Blood samples were obtained by drawing the blood into a syringe containing 5 ml. of the fluoride-thiosulfate solution, or if blood samples were taken by arterial or venous cannulae the blood was allowed to drip into a graduated cylinder containing 5 ml. of the fluoride-thiosulfate solution per 15 ml. of blood. The plasma was then separated by centrifugation and decanted. To the plasma was added an equal amount of the sodium acetate buffer, and this mixture was adjusted to pH 8.4 by the addition of a few drops of Na_2CO_3.

The columns used for the chromatographic isolation of the catechols were of the thistle tube type, having a bulb of 50 ml. capacity. The stem of the tube was approximately 23 cm. long with a constriction 5 cm. from the tip. The inside diameter of the stem was 5 mm. The columns were attached to a receiving flask through an adapter which was also connected to a vacuum source. The columns were prepared by placing a piece of glass wool at the constriction, a slurry was then made composed of 0.5 to 0.7 Gm. of the acid washed alumina and 2.5 ml. of acetate buffer. This slurry was poured on the column, washed down with 2.5 ml. of acetate buffer, and, after settling, mild suction was applied and so regulated that the rate of filtration was 20 to 30 drops per minute.

The plasma-acetate mixture was then added to the column, followed by 5 ml. of the acetate buffer and 5 ml. of glass-distilled water. These two filtrates were rejected. Occasional blocking of the column occurred due to precipitation of plasma lipid and protein constituents; this difficulty could easily be overcome by displacing the precipitate by means of a glass rod. The adsorbed epinephrine and norepinephrine were eluted by passing 5 ml. of 0.2 N acetic acid, followed by 5 ml. of water, through the column. To the acetic acid eluate was added 0.5 ml. of ethylenediamine dihydrochloride solution followed immediately by 0.7 ml. of ethylenediamine. The samples were then placed in a constant temperature water bath at 50° C. for exactly 20 minutes. At the end of this period, the samples were removed and placed in a cold water bath for 5 min. The samples were then saturated with NaCl (about 4 Gm.) and extracted with 6 ml. of isobutanol in a mechanical shaker for 4 min. If an emulsion formed during the extraction the samples were lightly centrifuged to break the emulsion.

Standard solutions containing 0.02, 0.04, 0.06, 0.08, and 0.10 micrograms of epinephrine in 10 ml. of 0.1 N acetic acid, a reagent blank and a column blank were also carried through the condensation and extraction procedures. With samples containing high concentrations of the catechols 0.2 μg of epinephrine was used to adjust instrument sensitivity.

The isobutanol phase was then transferred to the fluorometer cuvette and its fluorescence was determined.

Measurement of fluorescence: A Farrand Photoelectric fluorometer, Model A, was used to measure fluorescence. All fluorometer cuvettes were carefully calibrated and matched before use. Using Aperture No. 6 the sensitivity of the instrument was adjusted so that a reading of 100 was obtained on the galvanometer with the 0.10 microgram of epinephrine standard. The fluorescence of the remaining standard solutions, samples, and blanks was then determined with this sensitivity setting on the instrument. This procedure was followed using Corning filters 5433 and 3384 in the secondary and was repeated using Corning filter 2418 in the secondary. For both sets of readings the primary consisted of Corning filters 5113 and 3389. Standard curves were constructed by subtracting the reagent blank reading from the readings of the standard solutions and plotting these fluorescence values against concentration. Two curves were constructed, one for each secondary employed.

The concentration of epinephrine and norepinephrine in the samples was obtained by substitution of the appropriate fluorescence values in the following equations:

Using filters 5433 and 3384 in the secondary the ratio, epinephrine/norepinephrine fluorescence, is equal to 0.86. Therefore, the following relationship can be established:

$$A + \frac{N}{0.86} = b \qquad (1)$$

where A = concentration of epinephrine; N = concentration of norepinephrine; b = apparent epinephrine content of sample measured with filters 5433 and 3384 in the secondary.

Similarly, when measured with filter No. 2418 in the secondary the ratio, epinephrine/norepinephrine fluorescence, is equal to 4.22. Therefore,

$$A + \frac{N}{4.22} = c \qquad (2)$$

where c = apparent epinephrine content of the sample measured with filter 2418 in the secondary.

Solution of these simultaneous equations is as follows:

$$A + \frac{N}{0.86} = b \qquad (1)$$

$$A + \frac{N}{4.22} = c \qquad (2)$$

$$N = \frac{4.22 \times 0.86}{4.22 - 0.86} (b - c) = K (b - c) \qquad (3)$$

$$A = b - \frac{N}{0.86} = c - \frac{N}{4.22} \qquad (4)$$

The coefficients for the above equations were determined by constructing fluorescence curves of standard solutions of epinephrine and norepinephrine measured with each secondary employed. By dividing the slope of the epinephrine curve by the slope of the norepinephrine curve the ratio of epinephrine/norepinephrine fluorescence was determined. The ratio of epinephrine/norepinephrine using filters 5433 and 3384, which gave peak transmission at 510 mμ, was found to be 0.86 ± 0.10; using filter 2418, which has a sharp cut-off at 600 mμ, the ratio was found to be 4.22 ± 0.43. These constants will depend on the characteristics of the particular filters used and must be determined for each individual set of filters.

RESULTS

The precision and reproducibility of the epinephrine-ethylenediamine dihydrochloride condensation reaction was studied by preparing standard solutions of epinephrine in 0.1 N acetic acid and subjecting them to the condensation

reaction. The concentrations of epinephrine used were 0.02, 0.04, 0.06, 0.08, and 0.10 μg of epinephrine per 10 ml. of acetic acid. Duplicate samples of each concentration were analyzed in 11 successive runs. The results of this study are given in Table I. Plotting fluorescence against concentration results in a straight line indicating that the phototube response to the concentrations used is linear.

TABLE I. PRECISION OF CONDENSATION AND FLUORIMETRIC PROCEDURE WITH EPINEPHRINE

CONC. EPINEPHRINE (μg)	NO. OF DETERMINATIONS*	FLUORESCENCE (MEAN ± S.D.)†	S.D. (μg)	FLUORESCENCE (RANGE)
0.02	22	15.6 ± 2.8	0.004	6.9 – 21.0
0.04	22	28.4 ± 5.4	0.006	19.0 – 31.4
0.06	22	37.6 ± 4.0	0.008	32.0 – 44.0
0.08	22	49.5 ± 8.2	0.012	40.5 – 55.6
0.10	21	59.1 ± 5.7	0.008	52.9 – 65.1

*Duplicate samples at each concentration level were determined in each analytical run.
†Probability 0.05, 21 d. f., t = 2.08.

The same procedure and conditions were followed in studying the precision and reproducibility of the ethylenediamine dihydrochloride condensation with norepinephrine. The concentrations of norepinephrine used were 0.10, 0.20, 0.30, 0.40, and 0.50 μg. The results of this study are given in Table II. It can be seen that the fluorescence values for each set of duplicates lie within well-defined limits. Phototube response to these concentrations is also linear.

TABLE II. PRECISION OF CONDENSATION AND FLUORIMETRIC PROCEDURE WITH NOREPINEPHRINE

CONC. NOREPINEPHRINE (μg)	NO. OF DETERMINATIONS*	FLUORESCENCE (MEAN ± S.D.†)	S.D. (μg)	FLUORESCENCE (RANGE)
0.10	18	20.2 ± 2.36	0.013	15.5 – 26.1
0.20	18	37.2 ± 3.22	0.018	33.5 – 43.8
0.30	18	54.3 ± 7.91	0.046	42.5 – 59.6
0.40	14	69.9 ± 8.41	0.048	64.9 – 77.9
0.50	18	85.1 ± 2.51	0.012	80.9 – 88.7

*Duplicate samples at each concentration level were determined in each analytical run.
†Probability 0.05, 17 d. f., t = 2.11.

Mixtures of epinephrine and norepinephrine in 0.1 N acetic acid were also condensed with ethylenediamine. The fluorescence of the mixture was determined and the relative quantities of epinephrine and norepinephrine in the mixtures were calculated. These results are shown in Table III. These data indicate that mixtures of epinephrine and norepinephrine can be quantitatively assessed following condensation with ethylenediamine.

In order to evaluate the chromatographic step aqueous solutions of epinephrine and norepinephrine were adjusted to pH 8.4 with the acetate-carbonate buffer, subjected to chromatography on the alumina columns, and eluted with 0.2 N acetic acid. Quantitative recoveries could not be obtained; recoveries of epinephrine ranged from 10 to 70 per cent and recovery of norepinephrine ranged from 0 to 60 per cent. Since epinephrine is rapidly oxidized in alkaline

solution, the loss of epinephrine can probably be attributed to the lack of antioxidant in the solution. Quantitative recovery of epinephrine could be obtained from solutions containing 3 per cent bovine serum albumin which protected epinephrine after it had been adjusted to the alkaline pH. When serum albumin was used to protect the epinephrine, it could be recovered more completely when carried through the total analytical procedure than from untreated aqueous solutions. Although the per cent recovery covers a wide range (50 to 130 per cent), it was found that recoveries of graded concentrations of epinephrine from 0.02 to 0.10 μg were linear on a given day and that the quantity recovered was a function of the length of time the solution containing epinephrine was held at pH 8.4.

TABLE III. ANALYSIS OF MIXTURES OF EPINEPHRINE AND NOREPINEPHRINE IN 0.1 N ACETIC ACID BY CONDENSATION WITH ETHYLENEDIAMINE

SAMPLE NO.	EPINEPHRINE ADDED (μg)	EPINEPHRINE RECOVERED	NOREPINEPHRINE ADDED (μg)	NOREPINEPHRINE RECOVERED
1	0.010	0.013	0.040	0.035
2	0.010	0.008	0.040	0.042
3	0.020	0.022	0.060	0.072
4	0.020	0.019	0.060	0.057
5	0.030	0.031	0.030	0.024
6	0.030	0.033	0.050	0.056
7	0.030	0.027	0.030	0.032
8	0.030	0.028	0.050	0.053
9	0.050	0.049	0.020	0.019
10	0.050	0.044	0.020	0.023

Recovery of Epinephrine and Norepinephrine Added to Plasma.—In these experiments whole blood was obtained either by cardiac puncture or from the femoral artery of dogs anesthetized with Nembutal. The blood was collected in tubes containing sodium fluoride and sodium thiosulfate, the tubes were centrifuged, the plasma was removed, and either analyzed immediately or frozen until analysis was made. The recovery data for epinephrine and norepinephrine added to plasma are given in Table IV. Good recoveries were obtained with the added epinephrine, while the norepinephrine shows a much wider range of recoveries.

It has been reported by de Valk and Price[8] that epinephrine added to acetic acid passed over alumina gives a fluorescence which is approximately 150 per cent of its value in water or acid solution. We have examined this question and have shown that both epinephrine and norepinephrine condensed in acetic acid treated with alumina show an increase in fluorescence which is of a quantitative nature only.[10] That this phenomenon also occurs in plasma eluates was determined by adding epinephrine to column eluates of plasma and reading the resulting fluorescence against standard curves prepared in acetic acid and standard curves prepared in acetic acid which had been passed through an alumina column. Quantitative recovery was obtained when the fluorescence was calculated from the standard curves prepared in alumina treated acetic acid, while the "apparent" epinephrine content was increased from 20 to 30 per cent when calculated from the standard curve prepared in untreated acetic

TABLE IV. RECOVERY OF EPINEPHRINE AND NOREPINEPHRINE ADDED TO DOG PLASMA

ENDOGENOUS EPINEPHRINE (μg/10 ML.)	ADDED EPINEPHRINE (μg)	RECOVERED EPINEPHRINE (μg)	PER CENT EPINEPHRINE RECOVERED	ENDOGENOUS NOREPINEPHRINE	ADDED NOREPINEPHRINE	RECOVERED NOREPINEPHRINE	PER CENT NOREPINEPHRINE RECOVERED
0.012	0.020	0.029	90.6				
0.012	0.020	0.032	100.0				
0.019	0.020	0.039	100.0				
0.019	0.020	0.036	92.3				
0.076	0.020	0.111	115.7	0.017	0.080	0.071	73.2
0.076	0.020	0.111	115.7	0.017	0.080	0.071	73.2
0.076	0.020	0.110	114.5	0.003	0.080	0.077	92.7
0.076	0.020	0.099	103.1	0.003	0.080	0.082	98.7
0.012	0.040	0.044	84.6				
0.012	0.040	0.044	84.6				
0.076	0.040	0.128	110.3	0.017	0.060	0.048	62.3
0.076	0.040	0.117	100.8	0.017	0.060	0.066	85.4
0.076	0.040	0.115	99.1	0.003	0.060	0.045	71.4
0.076	0.040	0.123	106.0	0.003	0.060	0.063	100.0
0.012	0.060	0.090	125.0				
0.012	0.060	0.065	90.2				
0.019	0.060	0.084	106.2				
0.019	0.060	0.086	108.9				
0.076	0.060	0.143	105.1	0.017	0.040	0.047	82.4
0.076	0.060	0.153	112.5	0.017	0.040	0.040	70.1
0.076	0.060	0.150	110.2	0.003	0.040	0.034	79.0
0.076	0.060	0.140	102.9	0.003	0.040	0.040	93.0
0.012	0.080	0.087	94.4				
0.012	0.080	0.087	94.4				
0.019	0.080	0.100	101.0				
0.019	0.080	0.101	102.0				
0.076	0.080	0.171	109.6	0.017	0.020	0.026	70.2
0.076	0.080	0.170	108.9	0.017	0.020	0.023	62.1
0.076	0.080	0.173	110.9	0.003	0.020	0.022	95.6
0.076	0.080	0.175	112.1	0.003	0.020	0.013	56.5
0.019	0.100	0.126	105.8				
0.019	0.100	0.127	106.7				

Mean recovery (per cent): epinephrine, 103.8; norepinephrine, 79.1. Standard deviation of recovery (per cent): epinephrine, 9.4; norepinephrine, 13.08.

acid. The norepinephrine values were only slightly increased, the increase being less than 4 per cent. There is, therefore, an approximate 20 per cent loss of both epinephrine and norepinephrine during the analytical procedure, but if the concentrations of the catechols are calculated from a standard curve prepared with untreated acetic acid, the loss of epinephrine is compensated for and the values reported in this paper are a good index of the absolute levels of epinephrine in plasma. The norepinephrine values represent approximately 80 per cent of the absolute levels of norepinephrine in plasma.

TABLE V. EPINEPHRINE-NOREPINEPHRINE LEVELS IN NORMAL HUMAN SUBJECTS

SUBJECT NO.	EPINEPHRINE (μg/LITER PLASMA)	NOREPINEPHRINE (μg/LITER PLASMA)
1	0.00	6.82
2	0.00	5.60
3	0.15	1.09
4	0.00	2.41
5	0.06	3.43
6	0.00	0.00
7	0.00	1.00
8	0.00	1.80
9	0.00	0.00
10	0.00	1.20
11	0.00	1.80
12	0.00	0.00
Mean		2.09
Range	0.00-0.15	0.00-6.82

TABLE VI. EPINEPHRINE-NOREPINEPHRINE LEVELS IN MONKEY PLASMA

MONKEY NO.	EPINEPHRINE (μg/LITER PLASMA)	NOREPINEPHRINE (μg/LITER PLASMA)
M34-A	1.60	6.30
M32-A	1.69	3.72
M41-A	5.36	14.28
M45-A	1.00	3.40
M40-A	1.50	2.60
M52-A	0.60	9.00
Mean	1.95	6.55
Range	0.6-5.36	2.6-14.28

The analytical method described has been applied to the analysis of blood samples from dogs, monkeys, and humans. The dog samples were taken from the femoral artery of animals under Nembutal anesthesia. The samples from monkeys were withdrawn through indwelling venous catheters in conscious animals adapted to an experimental chair-type restraining apparatus. These data are given in Tables V to VII. In these 3 species, norepinephrine appears to be the principal catechol amine present in the blood. Both human and dog plasma show little epinephrine although significant amounts of epinephrine appear in some monkey plasma samples.

The spectral distribution of the fluorescence of the ethylenediamine derivatives of the catechols isolated from plasma of these species was determined using a Farrand recording spectrofluorometer and compared with samples of synthetic epinephrine and norepinephrine. These curves are shown in Fig. 1.

TABLE VII. EPINEPHRINE-NOREPINEPHRINE LEVELS IN DOG PLASMA

DOG NO.	EPINEPHRINE (μg/LITER PLASMA)	NOREPINEPHRINE (μg/LITER PLASMA)
1	0.00	1.30
2	0.00	0.00
3	1.20	3.30
4	0.00	0.00
5	1.37	9.50
6	0.00	5.00
7 (adrenalectomized)	0.00	4.50
8 (adrenalectomized)	0.11	1.10
9	0.15	0.15
10	0.26	8.05
Mean*	0.38	3.41
Range	0.00-1.37	0.00-9.50

Dogs under Nembutal anesthesia. Blood samples taken from femoral artery.
*Adrenalectomized not included.

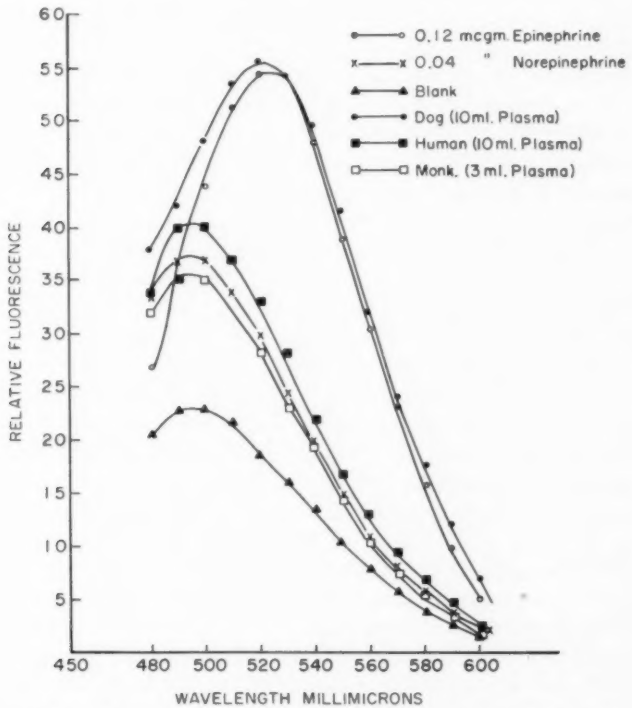

Fig. 1.—Comparison of fluorescent spectra of plasma samples with those of crystalline epinephrine and norepinephrine.

It can be seen that these curves closely approximate the emission spectra of the synthetic epinephrine and norepinephrine. In these particular human and monkey plasma samples no epinephrine could be detected and the emission spectra of these samples is the exact configuration of the emission spectra of

synthetic norepinephrine. The sample of dog plasma taken from the pooled plasma of an exsanguinated dog indicates that this sample contains predominantly epinephrine with some norepinephrine also present.

DISCUSSION

It is felt that the above data show the Weil-Malherbe and Bone method to possess a satisfactory degree of precision and reproducibility as judged on the following basis: First, repeated analysis of duplicate samples of crystalline epinephrine and norepinephrine, mixed in various proportions and carried through the condensation and fluorimetric steps, yielded quantitative recovery within a relatively narrow range. Second, the recoverability of crystalline epinephrine and norepinephrine added to plasma in varying amounts and carried through the entire procedure was similarly consistent within limits which would permit the detection of minute changes in concentration in samples from biologic experiments.

A second feature of the method considered in this study to a more limited extent is that of biochemical specificity. Although questions have been raised about the possibility that catechol amines other than epinephrine and norepinephrine may be detected by the method, no definitive evidence has been presented indicating that such substances exist in blood in sufficient concentrations to interfere with results. As described above, comparison of the spectral distribution of the fluorescence of the catechols isolated from dog, monkey, and human by this method with that of crystalline epinephrine and norepinephrine failed to show evidence of any interfering material in the plasma eluates. Recently Weil-Malherbe presented additional evidence to support the claim that epinephrine and norepinephrine were the only catechols present in plasma in sufficient quantities to be measured by this procedure.[5] The values for epinephrine and norepinephrine in human plasma are in agreement with those found by Manger and co-workers,[6] Aronow,[7] and de Valk[8] using various modifications of the Weil-Malherbe procedure, but are somewhat lower than those reported by Weil-Malherbe.[9] One possible explanation for the latter discrepancy may be that Weil-Malherbe uses plasma rich in platelets, which have been shown to contain appreciable epinephrine and norepinephrine.[13] Recent experiments in our laboratory have shown this method to be capable of reliably measuring changes in peripheral levels of epinephrine and norepinephrine associated with such manipulations as electrical stimulation of the splanchnic nerve or posterior hypothalamus[11] and intravenous administration of the crystalline hormones.[12]

The latter data strongly support our conclusion in the present study that the method is sufficiently sensitive and reproducible to warrant an exploration of its usefulness in a variety of physiologic studies. It must be emphasized, however, that in the average plasma sample one is dealing with minute quantities of extremely labile substances and that each operation in the analytical procedure, in the preparation of reagents, and in the cleaning of glassware must be rigidly controlled.

SUMMARY

A systematic evaluation has been made of the Weil-Malherbe and Bone method in an effort to determine its suitability for the measurement of epinephrine and norepinephrine in human, monkey, and dog plasma. Mean recovery of crystalline hormone added to plasma in concentrations ranging from 0.02 to 0.1 μg was 103.8 ± 9.4 per cent for epinephrine and 79.1 ± 13.8 per cent for norepinephrine. Measurement of normal plasma concentrations in human beings, monkeys, and dogs revealed readily detectable amounts of norepinephrine in all three species but appreciable epinephrine levels only in the monkey. It is concluded that the method is sufficiently reliable and sensitive for use in physiologic experiments.

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A NOTE ON THE SERUM IRON DETERMINATION DESCRIBED BY PETERS, GIOVANNIELLO, APT, AND ROSS

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SINCE the publication in August, 1956, of a simplified method for the determination of serum iron in this JOURNAL,[1] several persons have reported that they did not obtain single-phase solutions in the final step (addition of bathophenanthroline in 3:1 isopropyl/isoamyl alcohol). This of course prevents the obtaining of a reading in a spectrophotometer.

One cause for this difficulty has been found to be the use of trichloroacetic acid solutions which are weaker than the 30 per cent solution required. Another has been careless pipetting of reagents. However, in some cases no apparent cause could be found but, upon the suggestion of Dr. Rosario Maniglia of Hahnemann Medical College, Philadelphia, Pa., and Dr. Eugene W. Rice of Presbyterian Hospital, Pittsburgh, Pa., it was ascertained that decreasing the concentration of isoamyl alcohol in the color reagent would result in single-phase solutions.

Experiments have subsequently been conducted using varying proportions of isopropyl and isoamyl alcohols. While we always obtained single-phase solutions using the stated 3:1 ratio and using isoamyl alcohol (reagent grade) supplied either by Baker, Merck, or Eastman, we did find that increasing the isoamyl alcohol concentration from the stated 25 per cent to 31 per cent would give a multi-phase solution at 26° C. Upon decreasing the concentration of isoamyl alcohol, no difficulties were encountered between 0° C. and 40° C., and it was found that the isoamyl alcohol could be omitted entirely without altering the resulting optical density in either serum iron or serum iron binding capacity determinations.

Hence it is recommended that 0.02 per cent bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) in isopropyl alcohol be substituted for the bathophenanthroline in 3:1 isopropyl/isoamyl alcohol solution described in the original method. Bathophenanthroline dissolves very slowly even in alcohols, and the mixture should be allowed to sit overnight to ensure complete solution.

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From the Mary Imogene Bassett Hospital (affiliated with Columbia University), Cooperstown, N. Y., and the Laboratory Service, Boston Veterans Administration Hospital, Boston, Mass.

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STUDIES ON THE SEROLOGIC IDENTIFICATION OF ADENOVIRUS

I. Comparison of the Hemagglutination, Conglutinating Complement Adsorption, and Complement Fixation Tests

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RESPIRATORY disease caused by "adenovirus"[1] was first recognized by Rowe, Huebner, Gilmore, Parrott, and Ward[2] and by Hilleman and Werner.[3] The agents, although separable into specific serologic types according to neutralization data in tissue culture, contain a common complement-fixing antigen.

The present study was designed to compare the complement fixation (C.F.) reaction with a conglutinating complement adsorption (C.C.A.) technique and a hemagglutination test. The latter test was similar to the technique described by Friedman and Bennett[4] for detection of adenovirus antibodies.

MATERIALS AND METHODS

Preparation of Antigens.—Viruses were propagated in monkey kidney monolayer tissue cultures that were grown in 5-liter culture bottles. The infective titers averaged 10^{-5} for Type 3, 10^{-3} for Type 4, and $10^{-3.5}$ for Type 7. The media used consisted of medium 199[5] with 2 per cent horse or calf serum for cell growth and serum-free medium for virus propagation. The virus was harvested when the majority of the cells showed changes in morphology that were indicative of infection. All harvested fluids were stored at 2 to 5° C.

Raw, untreated tissue culture fluids were used as antigens for the C.F. and hemagglutination tests in some cases, but were found to be anticomplementary in the C.C.A. test. For the latter procedure it was necessary to dialyze such samples against phosphate buffer (10 volumes).

Two methods of concentration of antigen also were used. The first consisted of dialysis against polyvinylpyrrolidone (20 per cent). The original volume of raw fluid was reduced by this procedure to approximately one-tenth of its original volume. The concentrate was then diluted with veronal or phosphate buffer to about 25 per cent of the original volume.

The second method of concentration consisted of a methyl alcohol procedure similar to the first step of the poliovirus purification scheme of Schwerdt and Schaffer.[6] Tissue culture fluids were adjusted to pH 4.0, treated with 20 per cent methanol and 0.2 Gm. of Celite (Johns Manville) per each 100 ml. and the mixture agitated overnight. The Celite was collected on a glass filter, washed, and virus eluted with phosphate buffer adjusted to pH 7.2. The virus was concentrated approximately ten to fifteen fold.

Antisera.—Rabbits were injected, according to the method described by Rowe and associates,[2] with tissue culture fluids containing type-specific adenovirus. Animals were bled one week after the fifth injection and the serum separated and stored at -20° C. until used.

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Immediately prior to use, all sera were inactivated at 56° C. for one-half hour. Control serum was obtained from rabbits given a series of injections of fluid from uninfected tissue cultures.

*Hemagglutination Test.**—A modification of the Boyden technique was used.[7] Sheep erythrocytes were formalized according to the method of McKenna.[8, 9] To sensitize the formalized cells, one volume of antigen was incubated for 2 hours at 37° C. with two volumes of a 2.5 per cent suspension of tanned formalized cells in 0.5 volume of phosphate buffered saline at pH 6.4. The cells were washed in 1.0 per cent normal rabbit serum and suspended to a 2.5 per cent concentration in the same diluent.

Serial dilutions of antisera were made in 1.0 per cent normal rabbit serum, and 0.15 ml. of sensitized cells were added to each tube. End points were read after incubation for 12 to 18 hours at 1° C., and were recorded as the highest dilution of serum causing complete hemagglutination.

Conglutination Test.—The technique used in performing the conglutinating complement adsorption test has been described previously.[10] Both antigen and antiserum titrations were run and the source of conglutinating complement was unheated normal horse serum. End points were expressed as the highest dilution of antiserum or antigen showing inhibition of erythrocyte agglutination.

Complement Fixation Tests.†—The modifications of the Bengtson technique previously used for adenovirus were employed.[11-13] Suitable controls were included in all tests, and end points considered as the highest dilution of serum preventing hemolysis of erythrocytes. Block titrations were run to arrive at optimum antigen dilutions for the tests.

RESULTS

Typical results using 6 different antigen tests are shown in Table I. Of the 3 procedures, the conglutination test gave the most variable and least reliable results, probably due to the narrow range of optimum antigen reactivity. Similar results previously had been noted by one of us (J. E. P.), using the system for other antigens.

The hemagglutination test gave serum titers consistently higher than those of the C.F. procedure, and in some cases showed a tendency toward type specificity. This was also indicated by the results of Friedman and Bennett.[4]

There were marked differences between the different antigens used in these experiments. The PVP concentrated materials were of particular interest. All tests showed high reactions, but a high degree of nonspecificity was demonstrated with both complement-fixing procedures. However, the same material appeared to render the hemagglutination test more type specific.

Previous work has demonstrated that the C.F. antigen is a soluble fraction and not precipitated by centrifuge speeds necessary to sediment the virus particle.[14] Sedimentation studies were conducted to determine the nature of the hemagglutinating factor.

Raw virus, dialyzed material, and alcohol concentrated virus were centrifuged in a Spinco ultracentrifuge (No. 40 rotor) at the following rates: 5,000,

*This phase of the problem was conducted with the direct cooperation of Mr. John McKenna of the Research Laboratories Division, Merck & Co., Inc., West Point, Pa.

†We are indebted to Mr. Carl Newman of the Biological Control Department, Merck Sharp & Dohme, for these determinations.

TABLE I. ANTISERUM TITERS FOR ADENOVIRUS USING THREE SEROLOGIC PROCEDURES

ANTIGEN	ANTISERUM (TYPE)	HEMAGGLUTINATION	CONGLUTINATION	C.F.
Alcohol concen. Type 4	1	<2	>512	16
Alcohol concen. Type 4	2	<2	64	8
Alcohol concen. Type 4	3	16	64	64
Alcohol concen. Type 4	4	64	>512	64
Alcohol concen. Type 4	5	2	128	16
Alcohol concen. Type 4	6	<2	64	32
Alcohol concen. Type 4	7	16	128	128
Alcohol concen. Type 4	Control	2	<2	8
Raw fluid Type 3	1	<2	Neg.	<4
Raw fluid Type 3	2	4	Neg.	Neg.
Raw fluid Type 3	3	32	Neg.	<4
Raw fluid Type 3	4	<2	Neg.	<4
Raw fluid Type 3	5	<2	Neg.	<4
Raw fluid Type 3	6	128	Neg.	<4
Raw fluid Type 3	7	<2	16	Neg.
Raw fluid Type 3	Control	<2	Neg.	Neg.
PVP concen. Type 4	1	<2	256	64
PVP concen. Type 4	2	8	64	16
PVP concen. Type 4	3	<2	Neg.	64
PVP concen. Type 4	4	>512	>512	64
PVP concen. Type 4	5	<2	64	32
PVP concen. Type 4	6	<2	128	32
PVP concen. Type 4	7	2	>512	64
PVP concen. Type 4	Control	4	Neg.	16
Raw fluid Type 4	1	<2	Neg.	3
Raw fluid Type 4	2	<2	Neg.	3
Raw fluid Type 4	3	<2	Neg.	4
Raw fluid Type 4	4	128	16	4
Raw fluid Type 4	5	<2	Neg.	3
Raw fluid Type 4	6	<2	Neg.	3
Raw fluid Type 4	7	<2	Neg.	3
Raw fluid Type 4	Control	2	Neg.	Neg.

TABLE I—CONT'D

ANTIGEN	ANTISERUM (TYPE)	HEMAGGLUTINATION	CONGLUTINATION	C.F.
Alcohol concen. Type 4	1	64	Neg.	3
Alcohol concen. Type 4	2	16	Neg.	3
Alcohol concen. Type 4	3	128	Neg.	3
Alcohol concen. Type 4	4	256	Neg.	>24
Alcohol concen. Type 4	5	4	Neg.	4
Alcohol concen. Type 4	6	<2	Neg.	4
Alcohol concen. Type 4	7	128	Neg.	8
Alcohol concen. Type 4	Control	<2	Neg.	Neg.
Raw fluid Type 3	1	32	----	3
Raw fluid Type 3	2	<2	----	Neg.
Raw fluid Type 3	3	128	----	Neg.
Raw fluid Type 3	4	128	----	3
Raw fluid Type 3	5	<2	----	3
Raw fluid Type 3	6	2	----	8
Raw fluid Type 3	7	>512	----	3
Raw fluid Type 3	Control	4	----	3

10,000, 20,000, 30,000, and 38,000 r.p.m. for one hour. Supernatant fluids and resuspended sediments were tested for reactions. Table II indicates the speeds at which reactive material began to appear in the resuspended pellicle. The supernatant fluids contain the full amount of reactive antigen for all 3 tests, following centrifuging at speeds far exceeding those necessary for sedimentation of the virus particle.

TABLE II. SPEED REQUIRED FOR REMOVAL OF REACTIVITY FROM ADENOVIRUS SUSPENSIONS

ANTISERUM	TEST	RAW VIRUS SUSPENSION	DIALYZED VIRUS SUSPENSION	ALCOHOL CONCENTRATED SUSP.
Type 3	Hemagg.	>38,000*	38,000	30,000
Type 4	Hemagg.	>38,000	38,000	30,000
Type 3	Conglut.	-----	-----	30,000
Type 4	Conglut.	-----	-----	30,000
Type 3	C.F.	-----	-----	38,000

*Centrifuging speed per 1 hour.

DISCUSSION

A satisfactory serologic procedure is essential for the rapid identification of either adenovirus antigens or antibodies. Recognizing that the antigen used in

the complement fixation technique is group specific, it would be quite advantageous to obtain a type-specific analytical procedure. A second disadvantage of the C.F. test, at least with experimental animal sera, is its relative lack of sensitivity.

The C.C.A. test, while showing responses that are usually higher than those of the C.F. test, has disadvantages that render it unsuitable for routine laboratory use. The antigen concentration must be exactly optimum and must be determined prior to each test. This may be circumvented by selection of more suitable antigens. In an antigen dilution system, the test is more useful since the serum concentration in such an assay is not so critical. It is possible that a direct conglutination test[15] may be applicable and give consistent results.

The greatest promise for devising an improved serologic procedure is shown by the hemagglutination technique. Once the reagents are prepared, a rather large number of tests can be run in a short period of time. It also may be possible to increase the specificity of the test without losing sensitivity. The influence of various antigens on the specificity of the test presently is under study.

SUMMARY

A hemagglutination test and a conglutinating complement adsorption test have been compared with the conventional complement fixation test for the identification of antibodies to adenoviruses. The hemagglutination procedure appears to be satisfactory for antibody detection and has certain advantages over the C.F. test. The antigens for all 3 tests are apparently separable from the virus particle.

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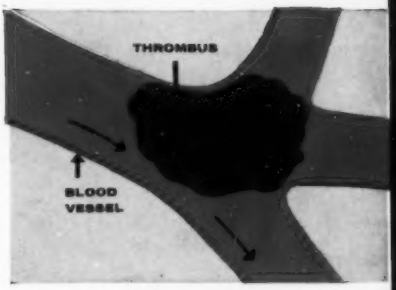
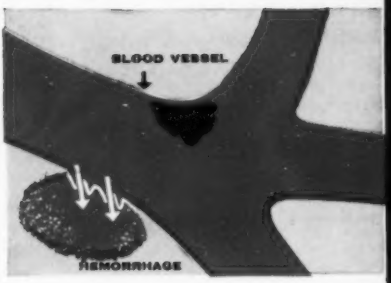
Erratum

In the article by DR. O. HERMAN DRESKIN, entitled "Prothrombin Measurement by Two-Stage Technique With Hemolyzed Whole Blood Thromboplastin," which appeared in the February issue of the JOURNAL, points 1, 2, and 3, under *Reagents* (p. 313) should read as follows: (1) 4.5 ml. of blood in 0.5 ml. 0.1 M sodium oxalate or sodium citrate. (2) Calcium chloride, anhydrous, 0.02 M (buffered to pH 7.3 to 7.4 by mixing calcium chloride 2.22 Gm., 0.1 M barbital sodium 60 ml., 0.1 N HCl 40 ml., and water sufficient to make 1,000 ml.). (3) Fibrinogen solution (prothrombin-free plasma).





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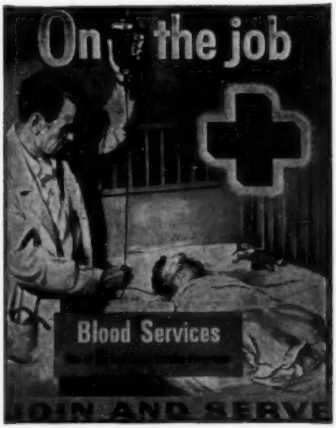

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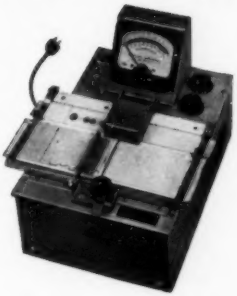

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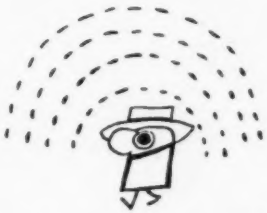

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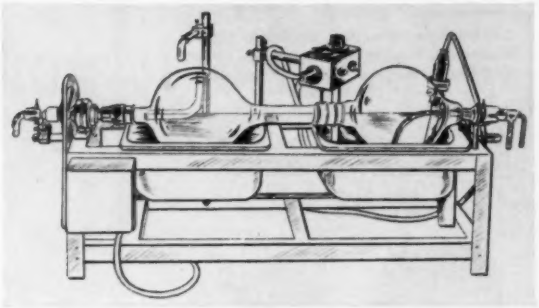

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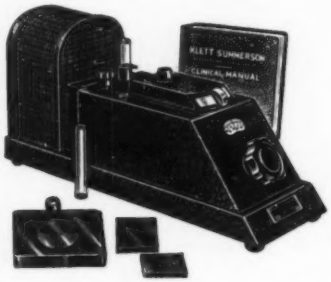

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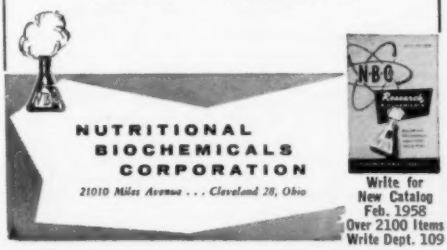

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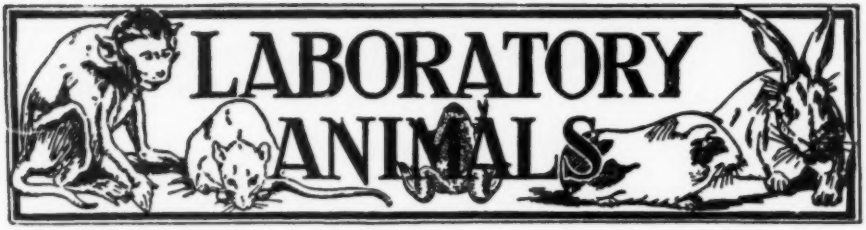

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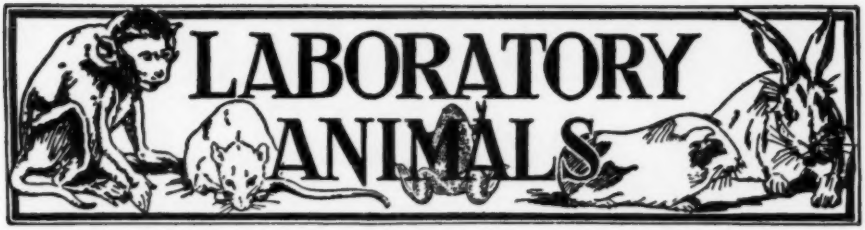

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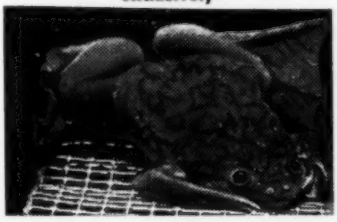

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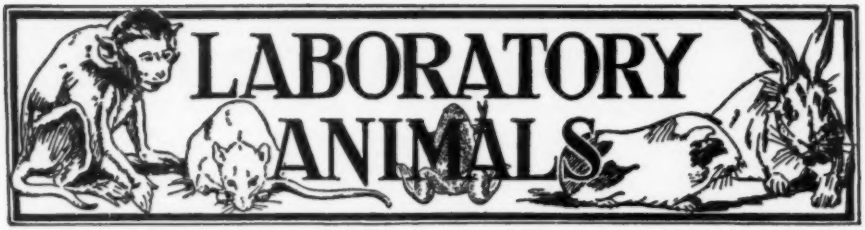

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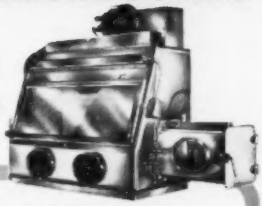

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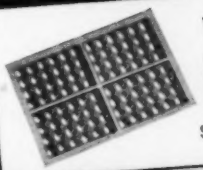

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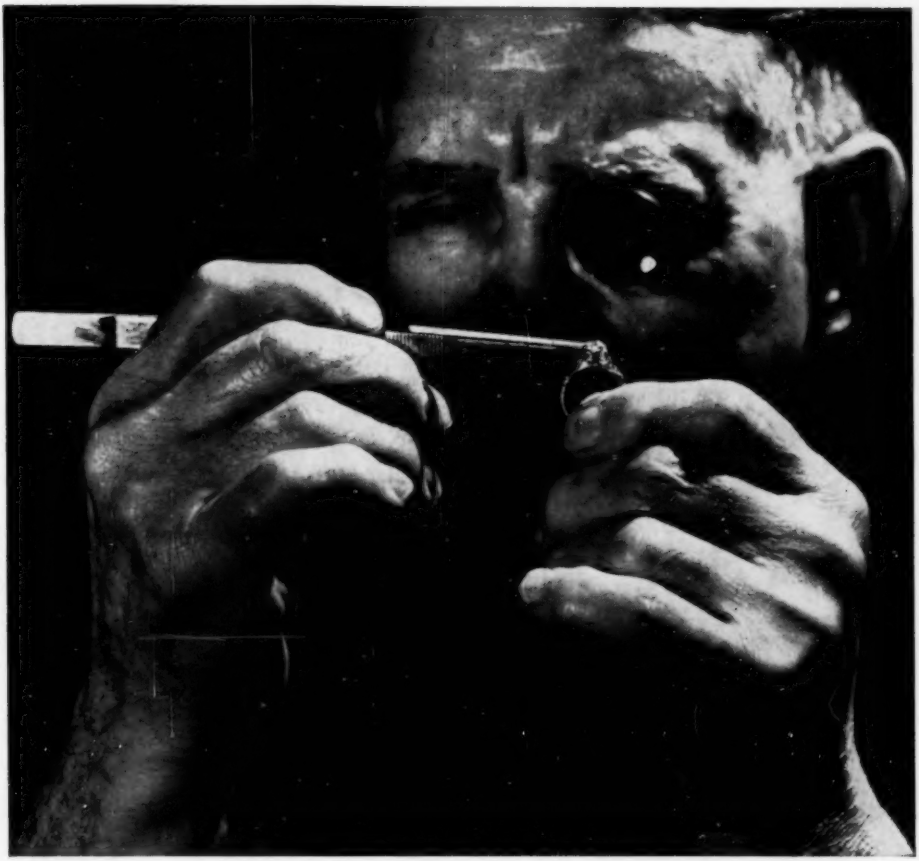

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